

The downfall of Mikhail Gorbachev

Pity poor Russia, the other quondam republics of the Soviet Union, the most talented and the most deprived part of the scientific community and also Mikhail Gorbachev (who may yet recover, but not quickly).

THE hard men of Moscow are not the only ones to blame for the unconstitutional sacking last week of Mr Mikhail Gorbachev from his post as President of the Soviet Union; he must also shoulder some of the blame. But only relatively little of it. Gorbachev has been a courageous and daring politician in the past five years. He has made *glasnost* and *perestroika* components of an international vocabulary. He has also edged the Soviet Union substantially towards constitutional reform — it is not an accident that his deposition came on the eve of approval of the new union treaty by at least five republics of the union — and has even committed his government to economic reform of a lasting kind. Even more important, he has demonstrated that the Soviet Union can abandon its prejudices against the outside world to the extent of signing treaties for the control of strategic arms, while Gorbachev's influence has given the satellite states of Eastern Europe a freedom they could not previously have expected.

These achievements have been marred in only three important ways — Gorbachev never had the courage to resign his post as general secretary of the Soviet Communist Party, he never took the risk of plunging for one of the economic programmes that might have worked and he allowed the Soviet army brutally to repress dissent in the Baltic states earlier this year. For the time being, it remains a mystery that a man of so much courage should have allowed compromises to dominate his short political life so far. Whether last week's events mark the end of his career is another matter. On the face of things, what is left of the Soviet Union cannot indefinitely manage without him.

Civil war, which has been on the cards for the past two years, is now the most likely outcome; too many of the republics — notably the Baltic states and Georgia — have gone too far down the road to independence easily to give up. Similarly, Mr Boris Yeltsin has done too much to assert the autonomy of the Russian republic for him easily to eat his words.

Meanwhile, we should all grieve for the condition of the Soviet people. That the economy has virtually collapsed is apparent from the empty shops, but this is only the most visible symptom of decline. It is even more significant that industrial production has fallen by more than 15 per cent in the past two years. Public services, once the Soviet Union's great pride, are meanwhile in

decline caused by under-investment and bureaucratic neglect. The research establishment — huge by any other standards, but under-productive for the past several decades — faces a still more miserable future. None of that will be made better if people choose to begin killing each other for the sake of a little freedom. Does the new Kremlin appreciate that truth? □

Electoral sustainability

The British Liberal Democrats are risking too much in their plans to create a green economy

To campaign successfully for election to public office by promising to increase taxes is usually an uphill struggle as Mr Walter Mondale found when, in 1984, he fought then Mr Ronald Reagan for the US presidency — and lost. The British political party called the Liberal Democrats, with which appear to rest the electoral hopes of some 15 per cent of the British population, seems entirely undeterred by Mondale's experience.

Last week, the party published a document called *Costing the Earth* which offers British people better environmental protection and what is called sustainable development in exchange for taxes on energy consumption and other devices. The advocacy of a grandiose unilateral policy of self-restraint on energy consumption and environmental damage is mistaken for at least two reasons (political expediency apart). First, the doctrine of fiscal neutrality is seductively over-simple. It is all very well to say that energy taxes would be offset by tax reductions elsewhere in the national budget, but would these fall in just the way that would preserve the competitiveness (such as it is) of British manufacturing? 742 The second obvious objection is linked with the first. Surprisingly for a party with a proper claim to international awareness the Liberal Democrats now pretend that Britain, acting alone on energy taxes and the like, can make a decisive contribution to global "sustainability" by acting unilaterally.

But that is a nonsense. It might be possible, by manipulating taxes in Britain, to drive energy-intensive industries elsewhere, but the Liberal Democrats would have been made a stronger claim on public attention if they had advocated international negotiations for the general adoption of their unilateral programme. □



Japan will fund major nanotechnology project

■ MITI shifting towards basic research
■ Linked to reorganization at Tsukuba

Tokyo

JAPAN'S Ministry of International Trade and Industry (MITI) has decided to launch a major government/industry initiative to develop nanotechnology. It is the latest and biggest in a series of Japanese projects aimed at building devices on the scale of nanometres.

MITI will submit a budget request to the Ministry of Finance at the end of this month to begin a large-scale (*ogata*) industrial project in fiscal year 1992. The ministry would like to plough ¥25,000 million (US\$185 million) into the project over ten years, with hopes that private industry will match up to 30 per cent or so of that amount.

Ogata projects are the largest in MITI's repertoire of industrial research projects and only one project is selected each year, so competition within the ministry to start new *ogata* projects is intense. The decision to support nanotechnology marks a significant shift by the industrial ministry towards the support of more basic research. Until now, *ogata* projects have focused on developing specific pieces of technology such as automatic sewing machines and hyper-sonic aircraft engines. But the Angstrom Technology Project, as the new programme is to be called, will aim at very basic research in the broad field of nanotechnology.

The project continues a government/industry trend here to develop nanotechnology. Earlier this year, for instance, MITI launched a 'quantum functional devices' project under its 'next generation' (*jiseidai*) programme for the development of basic technology for future industries.

The *jiseidai* project, headed by Professor Hiroyuki Sakaki of the Research Centre for Advanced Technology of Tokyo University, will receive MITI funding of about ¥5,000 million (\$40 million) over ten years. Twenty to thirty electronic companies, some of them non-Japanese, attended an explanatory meeting for Sakaki's project earlier this month in Tokyo. Between five and eight will be selected to join the project after submitting proposals. The number of companies joining the new *ogata* project will be an order of magnitude larger.

By setting out to support basic research in nanotechnology, the new Angstrom Technology Project is in many ways more like a *jiseidai* project than a typical *ogata* project, and the similarity is not

coincidental. MITI's Agency of Industrial Science and Technology has recently undergone internal reorganization so that a senior bureaucrat is now in charge of both the *jiseidai* and *ogata* projects. The ministry's purpose in making this move is to concentrate more effort on basic research and to try to remove some of the internal divisions that typically exist within Japanese ministries, MITI officials say.

The Angstrom Technology Project is also linked to a major reorganization of four MITI laboratories in Tsukuba Science City, where a new interdisciplinary centre will be created to carry out research on nanotechnology (see *Nature* 351, 90; 1991).

Over the past decade, about a dozen nanotechnology-related projects have been supported by the imaginative Exploratory Research For Advanced Technology (ERATO) programme run by the Research and Development Corporation of Japan, an affiliate of the Science and Technology Agency. Each ERATO project gets about \$10–\$15 million for five years to support a team of 15–20 young (under 35) researchers from academic institutions and industry led by a senior researcher.

Among the most recent are: the Aono Atomcraft project (1989–94), under the leadership of Masakazu Aono of the Institute of Physical and Chemical Research; the Tonomura electron wavefront project (1989–94), headed by Akira Tonomura of Hitachi, to develop applications of electron holography at the nanometre level; and the Sakaki quantum wave front project, in which a team led by Sakaki at Tokyo University will study the properties of semiconductor materials at the atomic level where electrons exhibit wavelike rather than particle behaviour.

The push by both ERATO and MITI to develop nanotechnology is not a planned or concerted government effort, however. Last week, officials in charge of ERATO were unaware that MITI is about to launch a major initiative in nanotechnology.

At present, it is mainly Japan's huge electronics companies that are showing interest in the Angstrom Technology Project. MITI officials are trying to interest biotechnology companies as well, but so far without much success. Representatives of biotechnology companies say they cannot see any immediate

application for nanotechnology in biotechnology because nanotechnology is concerned largely with surface phenomenon in two dimensions and cannot deal with the three-dimensional world of biotechnology.

Exactly what the project will focus on is not yet clear, and MITI officials have not yet decided on the detailed contents. The direction of the project should become clear next year when the project gets under way. As is always the case for *ogata* projects, MITI will apply for only a few tens of millions of yen in the first year. The budget request has to be approved by the Ministry of Finance and decision will not be made until the end of this year, but that is largely a formality. The biggest hurdle has already been overcome with the ministry's internal decision to support the project.

David Swinbanks

SOVIET UNION

Coup catches NASA engineers in Moscow

Washington

THE political uncertainty following the surprise overthrow of Mikhail Gorbachev is likely to put in question many planned collaborations between western and Soviet scientists, and the long-term effects will not be known for some time. But for a group of seven National Aeronautics and Space Administration (NASA) engineers in Moscow to coordinate the launch of the first US/Soviet space mission since 1975, the turmoil is all too immediate.

Last week saw the launch of the Soviet Meteor-3 satellite from the Plesetsk Cosmodrome, in the Russian republic, carrying a US-made ozone mapping device. NASA officials had planned to remain in the Soviet Union after the launch, studying telemetry signals from the satellite until its instruments are turned on, several weeks from now. But these plans have now been altered. Last Monday, Charles Cote, US project manager for the mission at the Goddard Space Flight Center, said his colleagues were sitting tight in their hotel. "Our main goal is to keep in touch with our people and get them back in this country as soon as possible," he said.

Shelby Tilford, NASA's director of Earth science, said that for the time being the US involvement in the mission will continue. "We'll deviate [from the plan] as appropriate, as things change."

The Meteor-3 satellite is carrying a Total Ozone Mapping Spectrometer, identical to the one on board NASA's Nimbus-7 satellite, which since 1978 has been monitoring the decline in the ozone layer. The joint mission is the first of a series planned under a 1987 US/Soviet agreement to cooperate in space exploration and science.

Peter Aldhous

FASEB rejects OSI rules

Washington

THE US Public Health Service Office of Scientific Integrity Review, which oversees the handling by the National Institutes of Health (NIH) of scientific misconduct investigations, has been overwhelmed by the response of the scientific community to its publication of rules for the operation of NIH's controversial Office of Scientific Integrity (OSI). But of some 1,800 responses received so far, it seems that only a handful are distinct comments. The vast majority of the responses were apparently modelled closely on a draft letter suggested by the Federation of American Societies for Experimental Biology (FASEB), a vigorous opponent of OSI.

OSI's rules were published for the first time in June (see *Nature* 351, 595; 20 June 1991), in an attempt to forestall further legal challenges to OSI, after a Wisconsin court declared the office's rules illegal. That decision centred on OSI's supposed violation of the accused's rights to 'due process', including the right to cross-examine witnesses. To formalize

OSI's procedures, the new rules stated explicitly that researchers under investigation may not confront other witnesses interviewed by OSI.

Late last month, Robert Cousins, the president of FASEB, wrote to the federation's members, urging them to protest against the OSI rules, and included a draft letter on which to base their comments. This letter catalogues a range of objections to the OSI rules, including the vexing question of due process for the accused and the lack of provision for sanctions against whistleblowers who make groundless accusations. FASEB would like to see OSI's role in misconduct investigations much reduced: "knowledgeable investigators with experience in the practice of science are in a far better position than bureaucrats" to weigh evidence of scientific fraud, the draft letter says. But few NIH observers believe that the Public Health Service, under pressure from Congress to increase OSI's resources, will respect FASEB's desire to lessen the influence of the office.

Peter Aldhous

Blast sets back H-II

Tokyo

JAPANESE engineers working through the night to develop Japan's next-generation H-II rocket suffered a serious setback during the early hours of 9 August when part of the rocket's main engine blew up during pressure tests and killed a 23-year-old engineer. The accident is the latest in a string of mishaps to hit the H-II. A series of fires during test firings of the rocket's main engine have already forced the National Space Development Agency to postpone the first launch of the rocket by a year to 1993. And to maintain the revised schedule, the agency's contractors are working around the clock — the accident occurred at 1:05 a.m.

Despite the accident, which occurred at pressures below the design tolerance of the engine, the space agency insists that it will maintain the present launch schedule.

D.S.

Commercial launch

REGARDLESS of the problems with the H-II rocket (see above), a private company that will contract out launches of the rocket has submitted bids to the International Maritime Satellite Organization (INMARSAT) for the launch of two satellites in 1995.

Rocket System Corporation, a consortium of 75 companies, last week submitted bids to INMARSAT's head office in London to launch marine communication satellites for the organization in July and November 1995. Company officials are confident the H-II will be operational by 1995. But they have requested the option to nullify any contracts if the development of the H-II is delayed.

Both INMARSAT and the International Telecommunications Satellite Organization (INTELSAT) have been encouraging Japan to submit bids for satellite launches using the H-II so that the organizations will have alternatives to the European Ariane rocket and US rockets (see *Nature* 350, 450; 1991).

D.S.

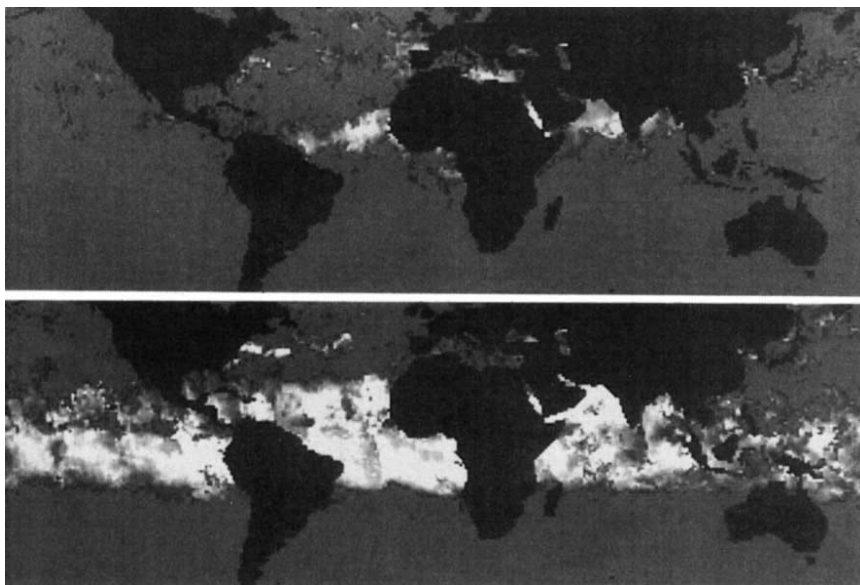
Wanted: astronauts

JAPAN'S National Space Development Agency is dismayed that only a handful of Japanese have applied to become astronauts for the Japanese module of the US space station, which is due to begin operations at the end of this decade. In 1985, when the agency recruited candidates to ride aboard the US space shuttle, more than 500 people applied for the three positions available.

But this time, one week after the deadline for applications, only 17 have expressed interest. Some agency officials blame the poor response on uncertainty over the future of the US space station. But an alternative explanation may be inadequate advertising.

D.S.

Before and after



These images from a US National Oceanic and Atmospheric Administration (NOAA) polar orbiting satellite illustrate the aerosol cloud thrown into the stratosphere by the eruption of Mount Pinatubo in the Philippines. Vernon Kousky from NOAA's Climate Analysis Center estimates that when the cloud becomes more evenly distributed through the stratosphere during the Northern Hemisphere winter, it may depress the mean global temperature by some 0.5° C. Judging by the effects of the last comparable volcanic event — the 1982 eruption of El Chichón in Mexico, which threw out about half the amount of dust as Pinatubo — global temperatures may be affected for the next two to four years, Kousky says. But, he adds, the effect will probably be undetectable because of the normal variability in climate.

The NOAA-11 satellite carries a high-resolution radiometer that can measure the reflection in the visible spectrum from dust and haze in the atmosphere, but only above the ocean, and only when the atmosphere below is cloudless. In the first image, compiled in early June, most of the reflectivity is from dust blown from the Saharan and Arabian deserts. The second, compiled over the week ending 25 June, shows the aerosol cloud from Pinatubo, spread into a belt surrounding the tropics.

P.A.

BTG has big plans for US

London

FOR the British Technology Group (BTG), it has been a year of living dangerously. Where just 12 months ago it rested in the safe embrace of UK government ownership and steadily climbing profits, BTG is now in the midst of a leap towards independence and an ambitious move into the United States. It has opened a new company — BTG-USA — to mine what its officials privately describe as a "vast, untapped market" of neglected US inventions, and at the same time it is orchestrating a massive political campaign to become a private company.

Last week, it released its first annual financial results since the opening of BTG-USA, and although the strain of growth is showing (setting up the US arm cut £3 million in profits off last year's figure of £9.5 million), it appears that the gamble may actually pay off. Nearly 80 per cent of BTG's £31 million income now comes from abroad, of which about half is from the United States. BTG-USA is about to announce its first agreement with a US university for a blanket technology transfer deal — a long-term profit-sharing arrangement with Princeton University to find buyers for the university's research. A recent survey by the technology transfer office of the Massachusetts Institute of Technology found that BTG has generated more patents and licences than all US university technology transfer offices combined. And in the midst of a recession, overall BTG revenue was still up by about 3 per cent.

This is heady stuff for a company that was set up in the 1940s by the government essentially as a marketing tool for UK universities. Since its modest creation, BTG has become the undisputed world leader in technology transfer — not, perhaps, as remarkable as it sounds, given that there is little competition.

With the exception of the smaller Arizona-based Research Corporation Technologies (RCT), no other major international technology transfer companies exist. "They're really our only competition in the world," says Gary Munsinger, RCT president. "I can see why they'd want to come to the United States."

If company estimates are right, BTG-USA can choose from a £10,000 million crop of undeveloped — but marketable — US technologies. Officials are unrestrained in their glee at entering what they see as a wide open market. "We intend to be as much larger than BTG-UK as the US economy is larger than the UK economy," is how Derek Schafer, director of the 16-person, £1-million US arm, put it at the launch of the US operation last September. "Expansion in

the US is the key to BTG's future," says Ian Harvey, chief executive.

For the time being, BTG-USA plans to place top priority on finding US takers for technologies that are already in its portfolio. The second priority — searching for new technology in the United States — will be focused first on US industry, and then on universities.

BTG argues that it is offering something that university and industrial technology transfer offices generally can not: a wide reach and a fierce defence. Without any special allegiance to any one industry, BTG can take technology farther afield than the in-house technology transfer offices of most US companies. And unlike most US universities, BTG has long and painful experience in protecting licences once they have been obtained. A six-year battle with the US Defense Department ended last year with BTG winning a \$6.1 million out of court settlement over unauthorized use of the company's Hovercraft technology.

Offered a chance to avoid some of those pitfalls, some US technology managers are already joining the BTG expansion, although they harbour no illusions about penetrating the European market. "We can't do everything," says Jean Mahoney, manager of the Princeton University technology transfer office that has signed a deal with BTG. "We're a small office and we need the help. I'm not particularly going after the European market; I'm just trying to sell our stuff."

Mahoney says that US government grants (the largest source of research funding at Princeton) often come with the stipulation that any products that come from the government-funded research must be offered to US companies first. Mahoney can now satisfy the government by giving US companies first choice, while still retaining a realistic European option if domestic buyers opt out.

Once parliament completes the legislative process to privatize BTG (something that, barring a political surprise, is expected this autumn, thanks to heavy BTG lobbying), its US prospects are expected to look even better. US government researchers, who are at present forbidden to make deals with a foreign government (a class in which BTG now falls), will then be able to offer up their inventions as well. The National Institutes of Health and the national laboratories of the Department of Energy have both expressed interest in establishing links once BTG is privatized.

Perhaps the best indication of BTG's bright prospects as it enters the US market is the manoeuvring that has already begun among potential investors.

Both the Wellcome Trust and the Nuffield Foundation have expressed interest in buying part of BTG, as have several UK investment and pension funds. Even the 48 UK universities, who have often complained of being forced by the government to give BTG the right of first refusal on their inventions, are trying to raise money to buy into BTG when it goes private. **Christopher Anderson**

AIDS

Meeting to move

Washington

The Harvard University organizers of the 8th International AIDS Conference planned for Boston in 1992 have decided to seek another location, outside the United States. At this year's conference in Florence, Max Essex of the Harvard AIDS Institute said the meeting would not be held in Boston unless the US government relaxed its controversial entry restrictions for people carrying the human immunodeficiency virus.

With no signs of movement on the issue from the US Administration, Essex says the time has now come to start making arrangements to hold the conference elsewhere. The conference now seems likely to take place in Europe.

Essex, who was to chair the 1992 meeting, has now handed over that responsibility to his Harvard colleague, Jonathan Mann, the former head of the World Health Organization's Global Programme on AIDS. "When I accepted the request to serve as chairman, . . . I didn't anticipate that any of these political questions would be in the limelight," says Essex, who is remaining as co-chair of the meeting for the scientific sessions.

Peter Aldhous

BRITISH SCIENCE

We shall be publishing on 12 September *Nature's* Manifesto for British Science, which will be offered without charge to any one of the political parties contesting the next general election and in the belief that it is an improvement on their own version.

Following publication, on Friday 13 September, there will be a public meeting at the Royal Institution, Albemarle Street, London W1, at which Sir Mark Richmond, chairman of the Science and Engineering Research Council, will be the principal speaker. The chairman will be John Maddox, editor of *Nature*. The meeting will start at 9.45 a.m. and end at 12.45 p.m. The political parties will be invited to send representatives, but there will be ample time for discussion.

Those wishing to attend should apply for tickets to Mary Sheehan, *Nature*, 4 Little Essex Street, London WC2R 3LF. □

Looking for alien life

Santa Cruz

SOVIET scientists have offered to help their US counterparts in the largest-ever Search for Extraterrestrial Intelligence (SETI), scheduled to begin on Columbus Day 1992 in honour of the 500th anniversary of Columbus's discovery of America. At an international SETI conference in Santa Cruz early in August, the dozen Soviet participants offered US scientists access to the Soviet Union's three largest radio telescopes.



The National Aeronautics and Space Administration (NASA) is very interested in the offer, said Jill Tarter, the radio-astronomer who heads NASA's SETI effort, but a formal agreement may take several years to negotiate. If an agreement is reached, it will be the first collaboration between US and Soviet scientists in this specialized, speculative field.

Although NASA has been designing and building SETI equipment since the early 1970s, the survey that begins next year will be its first major search, and in

PUBLIC HEALTH

New AIDS definition

Washington

THE US government's new definition of AIDS, announced in this week's *American Medical News*, could double the official number of people with AIDS in the United States.

The new definition, expected to be implemented by the end of the year, will count anyone infected with human immunodeficiency virus (HIV) and having a CD4 lymphocyte count under 200 as having AIDS. The old definition — filling a multi-page document — defines a person with AIDS as one who is infected with HIV and has one or more of several dozen designated AIDS-related conditions such as Kaposi's sarcoma. A person with a CD4 count below 200 is likely to be severely ill, but may not have any one of the designated conditions.

"The new definition will allow the CDC [the US Centers for Disease Con-

its first day of operation it is expected to dwarf all the smaller searches attempted over the past 32 years. The \$100-million, ten-year programme will include both a targeted search that examines 1,000 Sun-like stars within 100 light years of Earth and a sweeping sky search — a broader, less sensitive survey of the entire sky.

The targeted search will use several large telescopes — a 300-metre instrument in Arecibo, Puerto Rico, plus telescopes in West Virginia in the United States; New South Wales, Australia; and Nancy, France. It is this part of the programme in which Soviet collaboration could be helpful, as spacecraft-tracking telescopes in Evpatoria in the Crimea, at Ussuriysk near Vladivostok, and one under construction near Samarkand in East Asia would give NASA a much larger area for collecting signals.

In return, the Soviets would like to work with some of the state-of-the-art processors described by US scientists at the Santa Cruz meeting. The difficulty of SETI lies not so much in collecting signals as in determining their sources, and NASA has pushed for the development of new devices to distinguish between alien signals and run-of-the-mill interference from Earth. A processor designed by the Jet Propulsion Laboratory, for instance, will be able to scan half a billion channels for signals in the 1,000- to 10,000-megahertz range.

What happens if they detect an alien signal? "We'll leave it to the political science experts to decide when to respond and how," Tarter says.

Stephanie Sansom

trol] to count cases more accurately, and to find out into which populations the disease is spreading," says Charles Fallis, a spokesman for CDC.

At present, almost 70,000 people in the United States have AIDS according to the old definition. About 120,000 have already died from the disease. The CDC estimate that a further 150,000 people will fit the new definition of AIDS.

The old AIDS definition has come under increasing fire from AIDS advocacy groups and epidemiologists who claim that it fails to reflect the true magnitude of the pandemic. Many of the defining diseases were picked because they affected HIV-infected homosexual men. But as increasing numbers of women, intravenous drug users and children become infected, physicians are discovering new AIDS-related diseases.

Rachel Nowak

'Hacker' to go on trial

Canberra

FEDERAL prosecutors in Australia will call witnesses from the United States for the trial of a computer student accused of 'hacking' into sensitive computer systems at the US National Aeronautics and Space Administration (NASA) and the Lawrence Livermore National Laboratory in California.

Using a computer and a modem at his home in Melbourne, Nahshon Even-Chaim, a 20-year-old computer science student, allegedly altered research data at Livermore and disrupted operations at a NASA computer facility in Hampton, Virginia, isolating the computer for 24 hours. Also penetrated were computers at the University of California, the University of Wisconsin, Purdue University in Indiana, the private Texas company Execucom Systems Corporation and the Australian science agency CSIRO.

Last week, Even-Chaim was committed to stand trial on 48 criminal charges. The trial will be a test case for computer hacking laws introduced in Australia in 1989.

During his committal hearing, it was alleged that Even-Chaim gained access to computers through the worldwide computer network Internet by exploiting loopholes in the Unix operating system. The court heard that Even-Chaim considered the breach of computer security at the Livermore laboratory to be his chief accomplishment. Brett Wright

BIOTECHNOLOGY

Australian company near to a blue rose

Sydney

THE Australian biotechnology company Calgene Pacific estimates that it will be marketing blue roses by 1997, now that it has successfully isolated a blue pigmentation gene from other flowers. Such a rose cannot be produced by normal breeding techniques since roses do not have the necessary pigment.

The company says the blue roses will be sold initially at about US\$80 a stem.

Michael Dalling, managing director of Calgene Pacific, says the company has already developed an implantation technique for putting new genes in flowers by adapting a mechanism used by the crown-gall disease virus. It has used the technique to alter the genetic makeup of other flowers, he says. For instance, Calgene recently grew a batch of several thousand carnations with a gene for extended life. The company still has to marry the gene implantation technology with the new gene, which was isolated from delphinium flowers, but believes it will be able to do so within schedule, Dalling says.

Mark Lawson

Iraq's nuclear ambitions

Vienna

THE latest mission by the United Nations (UN) Special Commission has shed more light on the grandiose scale of Iraq's uranium enrichment programme. The money and the variety of technologies employed have brought comparison with the Manhattan Project. The inspectors' report confirms suggestions that Iraq could have had enough enriched uranium for nuclear weapons within 18 months. Despite the project's scale, it was not detected either by previous UN visits or by intelligence agencies before this year's Gulf War, raising questions about the stringency of the inspection agreements of the Non-Proliferation Treaty (NPT).

Mr David Kay, leader of the International Atomic Energy Agency (IAEA) inspection team, returned from Iraq only at the end of last week, says he is racing against Iraqi attempts to destroy the evidence of nuclear research. He speaks of "deception on a massive scale", with sites blown up, bulldozed and concreted over by the Iraqi military in between IAEA visits. The points marked "Location" on the accompanying map are those at which equipment was buried in the desert by the army. The IAEA's second mission was largely occupied with digging them out of the sand. Only with the recent fourth mission has the IAEA been able to fill in the main strands of the Iraqi project.

The main discovery was that Iraq was experimenting secretly with three parallel types of uranium enrichment: electromagnetic isotope separation (EMIS), centrifuges and chemical separation. The team also found production of small amounts of plutonium, and high-explosives research.

The main thrust of Iraq's research seems to have been in EMIS technology, at Al-Tuwaitha, a huge Iraqi research centre, where the IAEA had partial access before the war for NPT inspections. Installations of EMIS equipment were then found at Tarmiya and its twin Al-Shakart. The duplication of these sites shows the lavishness of the programme, and the determination to be invulnerable to Israeli attack.

The EMIS calutron equipment is essentially 1940s technology, "so old-fashioned we didn't realize what we were looking at for a while", says Kay. The calutrons separate the fissile uranium 235, around 0.7 per cent of the non-fissile uranium 238, by deflecting a beam of high-energy ionized uranium atoms through a vacuum chamber with electromagnets. The lighter isotope uranium 235 is deflected in a tighter trajectory than the uranium 238, and can be collected

separately. It is a relatively inefficient method; much of the uranium is lost on the inside of the vacuum chamber, and in practice the beams of the two isotopes may not be entirely separate. It is also extremely expensive in electricity; the Iraqi version required a power input of 100 MW, giving the lie to one Iraqi claim that it was being used to produce electricity.

Despite these difficulties, it is easier than many of the other uranium techno-

of-the-art centrifuge production centre. It would have been in operation late in 1991 and could have produced "400-600" centrifuges a year. A cascade of perhaps 200 would be needed for steady production of enriched uranium of weapons quality.

The team has not yet found evidence that Iraq had centrifuges up and running, nor that it had experimented with linking them in a cascade. According to the Iraqis, four were being tested at the research headquarters Tuwaitha, "in a building conveniently destroyed" says Kay.

Kay's team also found research into

chemical separation of uranium isotopes at Tuwaitha. The project was still at the laboratory level, mainly investigating the French method of extracting the isotopes by use of solvents. The IAEA believes that Tuwaitha had rejected a resin-based separation method under development by the Japanese because of the difficulties of getting hold of the chemicals without attracting attention.

Iraq was found to have processed a small amount of plutonium from its two small reactors at Tuwaitha. Iraq revealed that it had 2.26 grams in May, from irradiating fuel rods in the Soviet-manufactured reactor. According to Kay, these fuel rods were exempt from inspection by the IAEA under the NPT, and it was not a clear violation of the

NPT rules. However, the Iraqis later revealed, when the fourth mission arrived in July, that it had a further three grams. While the amounts are insignificant for any weapons programme (a bomb would need perhaps 8 kg), the three grams mark a greater infringement of the NPT rules as they were produced by irradiating material other than the uranium fuel rods in the core of the reactor.

The team found some evidence of research into high explosives. While there is no evidence that this research had led to production of weapons, the IAEA believes that "if they had made the decision to go to a nuclear weapons programme, then they would not have found high explosives a barrier".

The team found no evidence of any attempt to enrich uranium by gaseous diffusion, the method now used for large-scale production in the United States. The Iraqi explanation, for the moment accepted by the IAEA is that the technology of creating the barrier needed to distinguish the different isotopes posed certain technical difficulties.



In the second mission, near Tarmiya, the IAEA team intercepted a 60-truck convoy carrying pieces of the calutrons away. When the IAEA representatives approached they were shot at. They guessed the content of the trucks from aerial photographs, but established it for certain when they excavated pieces of the 24-ton magnets from the desert sand.

logies to create and operate, once a stable magnetic field is set up. Iraq would have had little problem finding descriptions in the open literature; much of the technology was declassified by the US government after the Second World War as other more efficient methods such as gaseous diffusion had taken over. Mosul, a site in the north of Iraq, had been equipped to supply uranium tetrachloride for the calutrons.

Nevertheless, Kay argues that it is wrong to judge the Iraqi calutrons by the present US standards. The IAEA believes that under the right conditions they could have produced around 30 kg of enriched uranium for Iraq in a year. The fourth mission established that the EMIS had just begun operation.

The team's second main discovery is that the Iraqi development of gas centrifuges to enrich uranium was much further advanced than suspected before the Gulf War. According to one of the team's advisers from British Nuclear Fuels Ltd, the Al-Farat site south of Baghdad is "better than Eurenco", the Dutch state-

AUSTRALIA

The scale of the project and the number of routes being explored at once have led to comparisons with the Manhattan Project, the US rush to devise nuclear weapons at the end of the Second World War. On IAEA estimates, assuming the project started in the late 1970s, it could have cost \$10,000–\$12,000 million, even allowing for low oil and labour costs.

The Iraqis have denied that they were trying to connect all these routes together, for example using the enriched uranium from the centrifuges to feed the calutrons to increase their efficiency. It looks more, according to the IAEA, as if they were pursuing all routes until they found a winner.

On IAEA estimates, through either the centrifuges or the EMIS, they would have had enough enriched uranium to equip a nuclear weapon within two years.

It seems unlikely that import restrictions have proved a big constraint, either on materials or choice of programme. It certainly would have needed foreign technical expertise in the centrifuge design. In this area the IAEA believes it has identified several names. Further missions will be needed to establish the scale of the centrifuge project. The IAEA expects to have finished by the end of 1991. However, removal of the fuel rods from the Tuwaitha reactors, at a cost of perhaps \$20 million, will take longer.

The scale of the activity previously undetected raises questions both for the intelligence agencies and for the IAEA in its role as watchdog of the NPT. The US military appears to have been unaware during the Gulf War of several of the largest sites uncovered by the IAEA. It was only after a first bombing of Al-Tarmiya military complex that aerial photographs gave a clue to the EMIS activity inside. Ash Sharkat, Tarmiya's twin, was not designated a high priority target in the war and was bombed, according to Kay, only because a pilot returning from Baghdad had unused bombs. Al-Farat, the centrifuge production centre, escaped entirely unbombed.

It would also be astonishing if the discoveries did not raise questions for the IAEA governors' meeting on 11 September. The last IAEA inspection was in November 1990. The IAEA, unsurprisingly, is not keen on the charge that it should have detected some of the programme in some of its twice-yearly inspection visits. It argues that its remit was simply to inspect the two reactors, one laboratory and one storage facility at Tuwaitha. It also pleads a lack of money. Its budget of \$60 million and 200 inspectors covers 1,000 reactors worldwide, and "Iraq's two small reactors were not its biggest priority".

Bronwen Maddox

Faculty pay up down under

Sydney

AUSTRALIAN universities should have a better chance of recruiting and retaining faculty, especially from overseas, now that their employees have won a major pay raise and are due a second, smaller increase over the next two years.

After years of decline of academic salaries in relation to other occupations in Australia, the central wage-fixing body, the Industrial Relations Tribunal, recently increased the pay scales for university staff by 12 to 16 per cent. And Australian academic unions are about to apply for further pay increases of up to 7 per cent. Under the rules for the latest round in Australia's centralized pay bargaining process, the Federated Australian University Staff Association and the Union of Australian College of Academics can expect increases of at least 2.5 per cent.

With the changes announced so far, a full professor's salary will increase by 12 per cent in stages to reach A\$76,000 (about US\$60,000) by July 1992. Increases for junior staff are better, with junior lecturer salary levels increasing by 10 to 20 per cent to between A\$40,000 and A\$47,500. Associate professors will earn from A\$59,000 to A\$65,000.

PLANETARY SCIENCE

Deep freeze for Galileo

Washington

THIS week, scientists at the National Aeronautics and Space Administration's (NASA) Jet Propulsion Laboratory will find out whether they have managed to free the Galileo Jupiter probe's main antenna, which has remained obstinately half unfurled ever since NASA first tried to open it in April. In the latest attempt to solve the problem last week, the craft was rotated, shading the antenna from the Sun for 50 hours to cool parts of the structure by as much as 100°C.

Fully unfurled, Galileo's main antenna looks like an inverted umbrella. But several of the carbon fibre ribs that support the structure have jammed, leaving it lopsided and functionally useless. This is a serious problem: if the antenna cannot be opened before Galileo's Jupiter rendezvous in 1995, most of the data from its 11 on-board instruments will never be relayed back to Earth.

NASA officials believe that cooling the antenna can solve the problem. The offending items are thought to be small metal alloy pins that keep the carbon fibre ribs correctly aligned, and project scientists hope that the differing thermal expansion of the two materials can be exploited to free the antenna. Last week's 50-hour

The unions say these increases are justified not only because of the relative decline in academic salaries but also because of major changes in both the university system and methods of allocating research funds.

The system changes include wholesale amalgamation of smaller college campuses, upgrading of several institutes to university status, a drive to get universities to earn more money from other sources and a major increase in the number of students in the system. While the last trend has resulted in overcrowding on campus and protests over campus conditions, one major surprise of the drive to attract students was a 13 per cent increase in university science enrolments. The increase occurred after several years of decline.

Another change, begun in 1988, has been to reduce the grants given to universities and redirect that money to the Australian Research Council for allocation in the form of grants to projects. This more centralized method of allocating research funds has emphasized larger projects and put more strain on junior staff, says Simon Marginson, research officer of the university staff association.

Mark Lawson

freeze is the second attempt to release the antenna by cooling — a similar manoeuvre last month, which lasted 32 hours, failed to do the job. Heating the antenna in the full glare of the Sun, which was tried in May, also proved unsuccessful. Programme manager Donald Ketterer, from NASA's headquarters in Washington, says that alternate cooling and heating manoeuvres will continue until the antenna opens.

The main antenna can relay 130,000 bits of information per second back to Earth, and with a back-up low-gain antenna capable of transmitting only 10 bits of information a second, a functioning main antenna is vital for the success of the \$1,600 million mission. Ketterer says that, without the main antenna, a single image from the craft's solid state imaging device would take several days, rather than a few seconds to transmit.

Jet Propulsion Laboratory scientists should know if the cooling manoeuvre has worked on Tuesday this week (20 August), shortly after *Nature* goes to press. If the jammed ribs have sprung free, telemetry signals from Galileo will reveal that a small wobble in the craft's flight, caused by the lopsided antenna, has stopped.

Peter Aldhous

Playing under new rules

Dresden

IF it had not been for German unification, Manfred von Ardenne, the renowned 84-year-old eastern German cancer researcher and polymath, might have been able to pursue his unorthodox theory of cancer treatment until his hundredth birthday, undisturbed by such Western trappings as peer review and open competition for research funds.

But, as in so many cases in the former East Germany, unification has changed everything. Now von Ardenne, a baron by birth who has been promoting his theories for more than 25 years with all the considerable (political) means at his disposal, is being asked to prove scientifically that his projects are worth supporting. He has no guarantee they will be approved. His change of fortune offers a revealing insight into the way research decisions were often made in East Germany and other Soviet bloc countries — and of how things have changed.

In Germany, von Ardenne's name is a household word virtually synonymous with 'inventive genius'. He left school at 16 without graduating, and he filed for his first patent — on a vacuum tube — a month later. Later, he left Berlin University after four terms to work on radio and the precursors of television, and in 1931 he built and demonstrated a crude early television set called a 'flying spot scanner'. He is generally credited with conceiving the scanning electron microscope in 1937, although the first working models were not constructed until much later, and not by him.

After the Second World War, he worked for the Soviets on isotope separation for the atomic bomb. For that, he received the Stalin Prize, worth several hundred thousand rubles.

Von Ardenne took advantage of the cash and his status as a Soviet hero to set himself up as a scientific power in East Germany. Buying several elegant villas in a smart area of Dresden, he established a private research institute — the only one in East Germany. There he initiated studies into electron-beam technology and vacuum tubes, work that gradually grew into a self-supporting business.

In 1964, bolstered by his successes in fields where he had no theoretical background or experience, von Ardenne launched into what would become his consuming passion: cancer research. He established a new section of his institute devoted to the development of cancer therapy based on the unorthodox theories of Otto Warburg, a 1931 Nobel laureate who had triggered von Ardenne's interest in the field.

The resulting three-step cancer therapy is controversial, to say the least. The

patient is first infused with a glucose solution to raise blood glucose to six times normal — a process that von Ardenne says acidifies tumour tissue and sensitizes it to heating. Next, the patient breathes a high-oxygen air mixture. Finally, the patient is heated until the core body temperature reaches 41.5 to 42.5 °C. This, von Ardenne says, preferentially kills the tumour cells.

Mainstream cancer researchers in Germany criticize the treatment. Typical is Oliver Lange of the Robert Janker Clinic, a private cancer hospital in Bonn, who describes von Ardenne's theories as more in the realm of religious belief than



Von Ardenne's prewar villa overlooks the Elbe river in Dresden.

science. (Lange stresses that this is his personal opinion and not that of the clinic, which is applying for money for a controlled test of some of von Ardenne's theories.)

Even within his own institute, von Ardenne is an embattled figure. "There always was a high turnover here," says Wolf-Karsten Mayer, a staff physician who came to the institute in 1986. "Highly qualified people especially usually left in a hurry," he adds, in part because von Ardenne insisted on publishing results before the experiments were complete or properly controlled.

Von Ardenne has an answer for all his critics. "They are uninformed," he says. "Or maybe they are jealous." He invites anyone to visit the institute and witness the success of the treatment for themselves. "I have over 400 publications," he says. "Maybe people have not had time to read them all." But that record does not carry quite the same weight as it would in the West. "Ninety per cent of [von Ardenne's] publications have ap-

peared in his own private publishing house," says oncologist Rolf Issels of the University of Munich.

Even institute staff members admit that a large majority of von Ardenne's publications appeared in East German journals of doubtful quality — journals to which von Ardenne had easy access.

As long as von Ardenne was working in East Germany, the criticism mattered little to him. He had a 'golden telephone' giving him access to the highest echelons of the government, Issels says. This access — and two million marks a year government support — allowed him to pursue his theories as he saw fit.

But times have changed. The unified German government, which has become interested in alternative cancer treatments, made it clear to von Ardenne that he will have to meet some stringent scientific and ethical criteria before he receives any more government grants.

So far, his institute as a whole has fared relatively well compared to other industrial research outfits since the collapse of the Communist system. It has shrunk from 500 to 220 employees, and the section for producing vacuum-tube equipment still needs a buyer or sponsor. But the section dealing with electron-beam technologies has been taken over by the Munich-based Fraunhofer Gesellschaft for the Advancement of Applied Research, whose approval is seen as a major compliment to the institute.

But this does not help the cancer research section. Although a new clinic for multi-step cancer therapy will provide some revenue, von Ardenne says if he does not get additional support soon, cancer research at the institute will be in serious trouble.

So, with the help of Western researchers and his own team of clinicians, von Ardenne is seeking the scientific proof he needs to qualify for government support. Wolfgang Scheef of the Janker Clinic in Bonn has applied for government money to test part of von Ardenne's treatment under strictly controlled conditions. And a team of physicians at the von Ardenne institute is in the midst of its own trial to determine the effectiveness of the treatment.

Researchers in both parts of Germany are following the saga closely. Although most expect the treatment to be at best a limited success when it is finally tested in a controlled way, they do not hide their respect for von Ardenne's ability to prosper under a variety of systems.

And if the treatment unexpectedly stands up to Western scrutiny, it will provide a final triumph to cap a long scientific career.

Steven Dickman

Response to Ptashne

SIR — I am writing to comment on Dr Mark Ptashne's letter (*Nature* 352, 101; 1991) which discusses two papers on which I am a co-author^{1,2}, and which relates these papers to the current controversy about the 1986 *Cell* paper by Weaver *et al.*³.

Ptashne's summary of the conclusions of our two papers is correct. However, it is not appropriate to infer that these conclusions fail to replicate the "central claim" of the 1985 *Cell* paper or that the papers support Dr Margot O'Toole's contentions regarding this "central claim".

First, the experiments that we performed were not designed to replicate the work of Weaver *et al.*, but instead to determine whether their results could be extended to a different antigen/idiotype system which involves entirely different specificities. The fact that we did not find antibodies entirely derived from endogenous genes but carrying idiotypic determinants characteristic of our transgene implies only that the findings of Weaver *et al.* cannot be generalized to other lines of transgenic mice with different specificity. Different transgenic mice having different recombined antibody heavy chain transgenes have shown remarkable variability in their phenotypes; thus the differences between our results and those of Weaver *et al.* should not be overconstrued. On the other hand, Weaver *et al.* speculated that some of their results might be explained by transgene isotype switching: our subsequent work has demonstrated clearly that such isotype switching can, in fact, occur^{2,4}. In this latter instance, con-

sistency between results from two different strains of transgenic mice (rather than the lack of consistency) is more meaningful.

Finally, the "central claim" of Weaver *et al.* is most directly supported by their results, summarized in Table 3 of the 1986 *Cell* paper, with cloned idiotype-positive hybridomas³. About 50 per cent of these hybridomas produced IgG or IgA rather than IgM. It is correct that our more recent work has shown that, in some transgenic mice, some B-cells can produce two separate mu molecules, one encoded by the transgene (mu-a) and one encoded entirely by endogenous genes (mu-b), and that these B-cells can secrete IgM heterodimers of the mu-a/mu-b type². However, as Dr Herman Eisen correctly indicates (*Nature* 352, 101; 1991), our observations of co-expression by IgM-producing cells are not relevant for the IgG-producing or IgA-producing hybridomas in Table 3 of Weaver *et al.*

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2. Rath *et al.* *J. Immunol.* **143**, 2074–2080 (1989).
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More on psycho-darwinism

SIR — Regarding Christopher Badcock's suggestion¹ that I might be reluctant to recognize my "freudian proclivities", in fact, I take considerable pleasure in the way in which psycho-darwinism² provides a means of tying Freud's idea of a death instinct back to the bedrock of evolutionary theory.

Some apparent difficulties are caused for psycho-darwinism by the phase in the human life cycle at which the onset of mental illness is most likely to occur, as M. S. Fazeli points out³. One possible explanation turns upon the exceptionally long period of nurturing that the human child requires. Because of this, in the natural state, the incapacity or elimination of a parent as a result of a mental disorder would, in almost all cases, seal the fate of the child, and genes common to both. Given that it is not always the most eligible partner who proves to be the most effective parent, there may be greater evolutionary stability in applying the relative success/failure test with greatest rigour well into the nurturing phase, rather than before reproduction.

Two other, mutually exclusive, possibilities are that: (1) it "pays" to rely

upon reproductive suppression, rather than mental illness and death, throughout the fertile period in case the individual eventually finds something it is relatively good at; or (2) in nature, natural selection operates on the incipient stages of mental illness, thus achieving elimination long before the mature phase we treat clinically.

Richard Dawkins' response⁴ is particularly useful because it has led me to develop psycho-darwinism in a way which makes it fully compatible with neo-darwinism. As he points out, unless I could somehow insulate my heirs from his, the inevitable outcome would be a Dawkins/Waller amalgam which could in no sense be represented as a total triumph of my genes over his. However, this conclusion does at least serve to define what constitutes 'triumph' in this context: achieving unaltered replication, generation after generation.

In terms of both my parable and the real world, there is only one group that meets this criterion: those few genes that are actually responsible for the existence and operations of the comparator mechanism which is central to psycho-darwinism. While themselves remaining virtually unaltered, they, in effect, use all other genes as mere resources to be permuted and re-permuted, selected and rejected, simply to ensure that they, the omnipresent comparator genes, are always on the side that is winning. Individual 'survival machines' are mere sub-agents, to be 'turned-on' when doing relatively well, and 'turned-off' when not. And, most cunning of all, it is the individual's own brain that makes the judgements, and triggers the appropriate electrochemical responses.

I have two reasons for suggesting that this final twist to psycho-darwinism makes it fully compatible with neo-darwinism. First, the comparator mechanism meets the requirement of acting solely in the interests of a specific group of genes. Second, the whole process is remarkably close to the extended version of inclusive fitness known as the 'green-beard' hypothesis, a description of which is to be found on page 207 of *The Blind Watchmaker*⁵. The "label", equivalent to a green beard, to which the bearers of the comparator mechanism respond is "an individual whom I have identified as aspiring to fulfil the same role as I do within my species", that is, a member of a peer group.

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Faux pas

SIR — John Maynard Smith's review of Robert Wesson's book *Beyond Natural selection* (*Nature* 352, 206; 1991) refers to the great French scientist "Jacob Monod".

I wonder whether this *coquille* derives from François Jacob and Jacques Monod being so often associated for their molecular biology work, or whether we must interpret it as an insidious attempt to decrease the number of French Nobel Prize recipients. Moreover, it would be unfair to reduce F. Jacob merely to J. Monod's first name.

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■ The error was made in the *Nature* office.
— Editor, *Nature*.

Deterioration of our cultural heritage

George Burns

Our cultural heritage is being destroyed faster today than at any time in the past. An understanding of the basic processes causing deterioration of ancient artefacts is urgently needed.

A FEW years ago, I visited an important excavation site. The morale of the usually noisy and happy members of the archaeological team was at a low ebb: although there had been good reason to believe that their site might yield important finds, the lengthy excavations had been fruitless. But the next day, enthusiasm was at its highest peak: a gigantic head of a statue of one of the most famous kings, whose images have been but rarely found, was discovered in the very last hours of excavations. Several experts volunteered to complete the excavation, without pay, and to document the find.

The find was considered so important that only members of the team were allowed to approach it. Nonetheless, I was sceptical about such precautions, believing the statue would soon crumble to dust. For my opinion, I was rewarded with an exquisite gaze of contempt, such as only a young humanist can bestow upon an arrogant scientist. My advice was ignored: neither the archaeologists nor their conservators seemed to be aware of the environmental shocks to which their find would be subjected. I recently revisited the site: the head had been removed and the site had been fiercely attacked by a hostile environment. There was no evidence of efforts to preserve the site or the antiquities still buried there.

History is full of similar incidents. Thus, E.A.W. Budge, an eminent Egyptologist who lived between 1857 and 1934 located an Egyptian papyrus. Such papyri, being fragile, have been found only rarely; those few that have been discovered are important sources of historical information. Budge placed his specimen in his strong-box and mounted it on his donkey. On arriving at his camp, he discovered that the bumpy ride had reduced his papyrus to a pile of dust.

Losses of such treasures seemed to be unavoidable in the past. But archaeology is today a professional discipline in a stage of unprecedented sophistication and growth. The archaeological team that found the head of the head of the statue was progressive in its thinking; few archaeologists even now would think of inviting a chemist, concerned with fundamental processes of matter, to visit their sites. Thus, the incident with the statue raises wider questions as to the

fate of excavated antiquities and sites. Was the incident symptomatic of a situation in which our excavated cultural heritage is being destroyed, possibly with participation of scholarly communities? If so, who is responsible, and what are the optimal, cost-effective remedies? To define the scope of these questions, I shall briefly consider a few important and representative cases in several countries where ancient monuments are venerated and where concern regarding their deterioration is unequivocal.

In Africa, the ancient Egyptian civilization flourished for about three millennia: according to UNESCO, about one third of all known antiquities are or have been located there. The Luxor area of upper Egypt is particularly rich in antiquities: the Karnak temples complex, built for 2,000 years between 2000 BC and the Christian era is its principal archaeological site, occupying more than a square kilometre (Fig. 1). For thousands of years these temples were affected by annual inundations of the Nile river but, because the monuments were unexcavated, water damage was minimal. After the monuments became exposed to the elements as a result of excavations, a canal had to be constructed around the entire complex to drain waters from inundated parts of the temples. When the Aswan dam was constructed, inundations became a thing

of the past; the drainage canal appeared to have become superfluous and fell rapidly into disuse. In the 1970s, a new type of salt deposit spread throughout the temples¹, causing disintegration of monuments and the demise of excavated antiquities^{1,2}.

In Asia, a major archaeological complex at Mohenjo-Dara, the centre of the empire of Harappans, in the valley of the Indus river, provides another example of deterioration of important excavated antiquities and sites. The Harappan empire once stretched from the Himalayas to the Arabian coast to Bombay, and was larger than that of ancient Egypt and Mesopotamia combined. Although Mohenjo-Dara was built between 2500 and 1500 BC, settlements in this area dating at least to the sixth millennium BC were discovered, making the Harappan civilization one of the oldest in the world. The excavation of Mohenjo-Dara, began in 1922, had to be largely interrupted because the unearthed structures deteriorated rapidly and in some cases crumbled away.

In Mesoamerica, the discovery about 50 years ago of the Bonampak temple complex yielded for the first time a temple with a completely painted interior with murals of high artistic quality, providing a rare glimpse of the traditions of the Classic Mayan civilization of the late eighth century. Although the murals

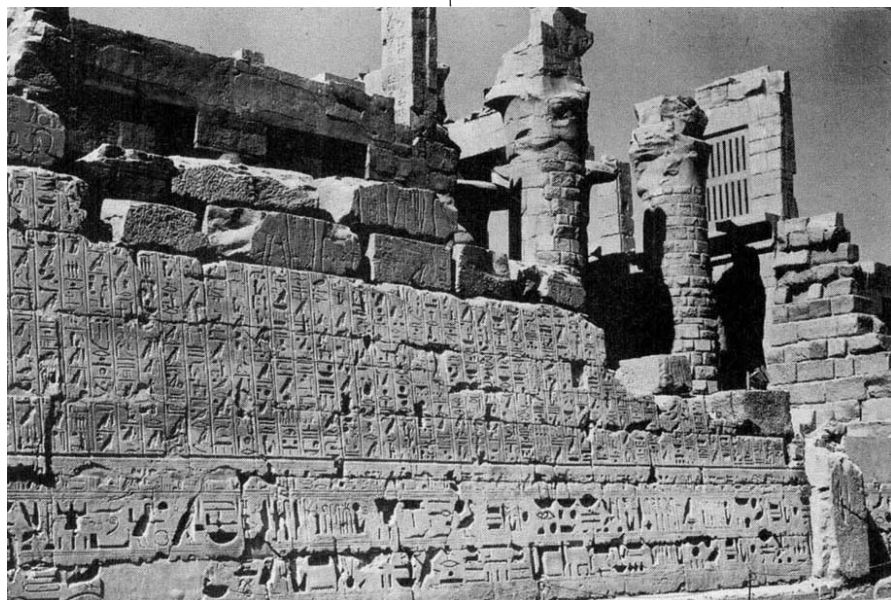


FIG. 1 The Kanak temples complex. On the walls of these temples history of millennia were inscribed.

were originally in good state of preservation following the discovery, environmentally induced deterioration is now occurring at an alarming rate³.

In northern Europe, there are fewer excavated antiquities that are older than one millennium; generally, these antiquities receive much care. Even there, however, there is evidence of rapid deterioration. Thus, the famous Lascaux caves have been closed to visitors for the past 30 years, and a replica cave has been constructed for tourists.

Even the best-known excavated antiquities may not escape rapid deterioration. A bronze statue of Marcus Aurelius survived for centuries in the polluted waters of the Tiber until it was recently discovered and installed in honour on Capitol Hill in Rome. Its deterioration in its new environment was so precipitous that it had to be placed in a protected atmosphere in a museum. Even museums are not always safe, being subject to overcrowding and exposed to fumes and dust of cities — such is the threat to the famous Egyptian National Museum in Cairo, a city that has quintupled its population in the past 15 years.

There are many other examples of deterioration in various parts of the world (see, for example, refs 3–7). Taking into consideration the recent, near-exponential rise in archaeological explorations and the number of newly excavated sites, one can readily see that today the magnitude of the demise of cultural heritage throughout the entire world is unparalleled. Perhaps the most telling testimonies come directly from archaeologists, who almost routinely express their genuine dismay about 'after-care' of monuments, and even suggest that in some cases sites should be better left unexcavated.

This deterioration is particularly evident in some developing countries. Generally, these countries greatly value their cultural heritage, not only out of national pride but also as one of the platforms upon which a new technological society can grow. Therefore, according to governments of some of these countries, excavations which are conducted by archaeologists from the west, if pursued without an adequate back-up ensuring preservation, amount to what is now often referred to as 'archaeological imperialism'. Such factors add complications to an already serious situation: they provide yet another cause for international tension, often in sensitive regions of the world.

Much of our cultural heritage is now being destroyed through wars, natural disasters, grave looting, destructions of rainforests, factors which are outside the domain of science. But this cannot justify deteriorations of cultural heritage in

which scholarly activities are implicated.

Who, or what, is the guilty party? In some cases the accusing finger has been pointed at the science of archaeology itself. In Turkey, for example, archaeologists routinely have to provide a guarantee that their finds will be adequately protected after they are excavated. Such conditions complicate research: archaeologists can claim with justification that studies of deterioration processes of antiquities is not a part of their subject. Archaeologists in the past were not considered responsible for preservation of their finds, although many of them attempted to do everything possible to save them. How can one feel now justified to expect a fundamental change of an already demanding discipline, particularly at a time when financial support is meagre?

Should archaeometrists, archaeological scientists and archaeological chemists take some responsibility for the failure to preserve our cultural heritage? After all, it is they who are supposed to have all the scientific knowledge to be used for studying ancient materials. It is true that archaeometrists undergo periodic soul-searching to define their own responsibilities⁸. Occasionally they even go as far as to accept the possibility that they "should recognize our responsibility to understand the mechanism of decay of (excavated) objects" (page 69 of ref. 8). But for the most part, the mandate of an archaeometrist is "to move from a dominant position (of a scientist) to a subservient one — of a humanist" (page 51 of ref. 8). Consequently, there seems to be little evidence that archaeometrists have become involved with the problems of deterioration of the cultural heritage (see, for example, ref. 7).

It would perhaps be even more unfair to accuse conservators and restorers for failure to provide adequate protection. These specialists, unsurpassed for "precision and patience of the artisans who first tried their hands at the job" (see ref. 7) have been trained to deal largely with conservation of individual monuments or works of art, rather than global problems of massive deterioration. Although the need for them to become involved in fundamental research has been acknowledged, little or no progress has been made⁹.

An easy target for accusation are the governments and administrations who are in charge of excavated antiquities: indeed I was initially inclined to pass such a judgement. But my view changed when I observed one senior administrator in a developing country defending vehemently the cause of preservation of cultural heritage to his superiors, to the point that his entire career became jeopardized.

The responsibility for the demise of

our ancient heritage results, I believe, from inadequate recognition of the problem and its magnitude. Unless the situation is recognized and action is taken, future generations may look back at the present time as that when the cultural heritage of the human race was most extensively damaged.

Everyone is dismayed at the disappearance of cultural heritage. Specialists of all disciplines involved recognize that care of excavated antiquities and sites has been inadequate and that the problem is cumulative, continuous, near-exponentially progressive and dangerously extensive. All agree that money is badly needed, but that there is little hope that it will be forthcoming from poorer countries, where much antiquity is located. Some form of international arrangement is needed. Up to this point, there is agreement among all concerned. But different views are expressed whenever practical steps toward resolution of the problem are considered.

Archaeologists recognize that they may have to reduce their scale of excavation because an extensive demise of cultural patrimony is unpalatable and intolerable for a reputable discipline. Presumably, funds unspent because of a reduction of the scale of archaeological field work could be allocated for preservation of finds and abandoned archaeological sites. Such an approach is painful to accept for any active, vigorous discipline, for it involves a conscious decision to curtail its own activities. Furthermore, under such a scheme, as the number of excavated sites increases, archaeology will lose progressively and cumulatively its funding, as it is syphoned off to protect cultural heritage. In any case, it is hard to see how any 'unspent' money could materialize in a field which is eternally short of funds. Alternatively, archaeologists often 'accept the inevitable' and pronounce that much of our cultural heritage will be lost anyway. People with this view believe that archaeologists should save a few national treasures and let the rest be lost — provided, of course, that all relevant information is properly recorded, documented and studied.

Today, both these approaches are in vogue, and documentation of information on excavated antiquities is an important part of archaeological work. Thus, the epigraphers of the Luxor and Karnak temples resurrect meticulously on paper the semi-obliterated hieroglyphs of the original monuments. Furthermore, many promising archaeological sites still remain unexcavated. For example, an enormous temple of Amenhotep III, of which only the colossi of Memnon are still towering above the ground, remains untouched by archaeologists (Fig. 2). Elsewhere, about three-



FIG. 2 The temple of Amenhotep III with colossi of Memnon, general view.

quarters of Mohenjo-Daro is still buried.

Restorers and conservators are making remarkable progress, yet their work is often handicapped because the fundamental chemical processes responsible for the deterioration are site-specific and often not understood. Consequently, in some cases, their work may even accelerate the demise of monuments (see, for example, ref. 4). Furthermore, the sheer size of the problem is often daunting: how can one attempt to conserve acres of deteriorating antiquities at such enormous sites as Karnak or Mohenjo-Dara?

Although there are several disciplines concerned with the salvage of cultural heritage, this goal is not usually central to them. Thus, archaeologists are generally concerned with purely archaeological problems, and environmental scientists, who recognize deterioration of cultural heritage as an environmental problem, are concerned that the archaeological aspects lie outside their field of expertise. The demise of our cultural heritage is due at least in part to the multidisciplinary of the problem and to the weakness in links between disciplines¹⁰. Work directed at strengthening these links, especially the weakest and the most crucial ones, seems promising. In general, the weakest links among various disciplines exist if these disciplines are furthest away from each other in terms of the interest, outlook and educational background of their practitioners. Among the disciplines concerned with the demise of cultural heritage, the widest gap is perhaps between archaeology, a central field, and those physical sciences which can deal with the fundamentals of the deterioration processes of antiquities.

I have argued the value of establishing the subdiscipline of environmental archaeological sciences, or eco-archaeometry^{10,11}, which deals with studies of fundamental physicochemical phenomena which lead to deterioration

of ancient materials. Once harmful processes are understood and quantified, an eco-archaeometrist can identify the most cost-effective ways to protect the cultural heritage. One of the basic assumptions is that deterioration processes must be understood on a fundamental, molecular level so that efforts of conservators and archaeologists are not misdirected. Eco-archaeometry has to rely on 'high-technology' techniques such as advanced analytical methods, physicochemical approaches and computer modelling¹⁰⁻¹².

From the viewpoint of the eco-archaeometrist, archaeological explorations thus need to be preceded by an investigation of physicochemical processes and change of rates of these processes at a potential site. Eco-archaeometry would promote 'rescue' archaeology while discouraging excavations at environmentally stable sites. Even if these sites are promising archaeologically, excavations can be postponed for as long as necessary. An eco-archaeometrist places less emphasis on the excavation of archaeological monuments *per se*, but rather ascertains whether excavation would not be detrimental to excavated monuments or sites. Yet archaeology and eco-archaeometry are not on a collision course: each discipline has something to offer the other. Their symbiotic co-existence is beneficial to excavated antiquities and archaeological sites.

The work of an eco-archaeometrist can suggest the change of direction of work at existing sites. It was eco-archaeometry that made it possible to show that the salinity of the underground waters at Karnak is due to high evapotranspiration at nearby irrigated fields^{1,2}. Once the salinization process in the Karnak area were understood, it became clear that the canal separating the temples from nearby irrigated fields should be cleaned, deepened and reactivated rather than abandoned and filled up to its rims with refuse and even with archaeological debris. The desalinization

programme at Karnak has now begun, and environmentally detrimental excavations have been postponed.

In Egypt, there has been a concern about deterioration of the tomb of Nefertari, Valley of the Queens, where the most beautiful murals are now in an extremely precarious state. While a superb job on emergency restoration of these murals was recently completed, our eco-archaeometric study concluded that the murals survived for 3,250 years only because of the extraordinary climatic stability of the tomb. Once these conditions were quantified^{11,12} it was possible to propose a long-term plan¹² for preservation of the tomb, involving the construction (now in progress) of a replica tomb nearby for tourists and construction of a climate control station to stabilize temperature, humidity and biological activity within the tomb to allow limited visits.

At Mohenjo-Dara, the principal effort for preservation of monuments seems to come from agencies planning to divert the Indus river, thereby lowering the water table by about 60 feet. Although this would make excavations easier, an eco-archaeometrist would be concerned that antiquities would thus become desalinized and reduced to dust. At Lascaux, the eco-archaeometrist would be concerned with the nature and rates of decay of the murals caused by environmental stress.

The holistic approach of eco-archaeometry allows the natural scientist to investigate a problem in a way often overlooked by archaeologists. Because eco-archaeometry provides basic information, cost-effective decisions can be made about whether to invest in archaeological or rescue projects (which are usually expensive) or whether such plans should be postponed or altered. I believe that the use of this approach will contribute to the protection of our cultural heritage and prevent much of its deterioration. □

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The social return of academic research

A careful economic study reveals that academic research is an excellent investment but the most famous universities may not be the best to invest in.

FOR years, US scientists have justified receiving government support by arguing that whatever investment is made in basic research will eventually be returned many times over in benefits to society. But despite the plausibility of this contention, there has never been any proof — until now.

Edwin Mansfield, director of the Center for Economics and Technology at the University of Pennsylvania, has examined the question directly by asking US industry which new products and processes could not have been developed without academic research.

The answer, published earlier this year (in *Research Policy* 20, 1–12; February 1991) confirms what scientists have said all along: academic research more than pays for itself when its economic contributions to society are taken into account. More recently, however, Mansfield has asked exactly which academic research is most valuable to industry, and these results hold a few surprises. His still-unpublished data show that the most valuable research is not always that done at the best schools or in the large, well-funded research groups. Although he warns that his work is too preliminary to base research policy on, it seems certain to generate discussion and debate over the role and funding of research.

Mansfield chose a random sample of 76 major US companies in seven industries — information processing, electrical equipment, chemicals, instruments, drugs, metals and oil — and asked their executives what percentage of their new products and processes commercialized from 1975 to 1985 could not have been developed (without substantial delay) without academic research carried out in the previous 15 years.

The results were impressive: 11 per cent of the products and 9 per cent of the processes had depended on academic research. The percentages varied from industry to industry, with drugs (27 per cent of products and 29 per cent of processes) at the high end, and oil (1 per cent of each) at the low end.

He also found that he could put an approximate dollar figure on the value of academic research to industry and to society as a whole. The bottom line, Mansfield calculated, is that investment in academic research has an average annual rate of return to society of about

28 per cent. Although the precise details are complicated by such factors as an average lag time of seven years between research and commercialization, this pay-back is equivalent in economic terms to putting money into a saving account that annually pays 28 cents on the dollar.

And that is a conservative estimate, says Leonard Lederman of the National Science Foundation, who encouraged Mansfield's work and followed it closely. "He did everything possible to estimate the cost of academic R&D as high as possible, such as including costs for research that was never intended to have industrial payoffs," Lederman says. "He also estimated low on the return." The result is a rough figure, but one that should, if anything, understate the value of academic research.

Mansfield next went back to the same companies to determine exactly what types of academic research were of greatest worth. He asked each of them to name five researchers whose work during the 1970s and 1980s was most valuable to it. Some of the details were predictable, but some were unexpected.

Most predictable were the areas of research of greatest worth to different industries: electronics companies most often cited research done by electrical engineers; information processing companies preferred computer scientists; chemical companies, chemists; and so on. Pharmaceutical companies were the most catholic, pointing to biology, chemistry and pharmacology departments.

Many of the schools producing the most cited research were also predictable: Harvard, the University of California at San Francisco, Stanford and Yale led the list for pharmaceutical companies, and the Massachusetts Institute of Technology and the University of California at Berkeley rated top for both electronics and information processing.

But then there were some surprises. Washington University and the University of Utah were first and third among those cited by the chemical industry, although neither school was in the top 12 on a 1982 list of the country's best chemistry departments compiled by the National Academy of Sciences. The University of Illinois was mentioned more often than Stanford by companies in both electronics and information processing.

Intrigued, Mansfield compared industry's rating of university departments

with the quality of the faculty as determined by the Academy. He found varying degrees of agreement between the two measures — strongest in electronics, almost nonexistent in chemistry — but on the whole, he says, industry does not seem to favour the most respected departments as much as one might guess.

Two possible explanations come to mind, Mansfield says. Some otherwise undistinguished departments may have a few very good researchers whose work is valuable to industry. And researchers in the 'better' departments may concentrate on work that has payoffs further than 15 years in the future.

Part of the explanation also seems to be that companies tend to support research at nearby universities, whether or not the schools are highly rated. In turn, the companies are more aware of the research going on at these local universities and more likely to use it in their product development.

Mansfield found that nearly 40 per cent of the researchers cited by electronics and information processing firms worked in the same state as the company that cited them, as did about a quarter of the researchers listed by pharmaceutical and chemical companies. Part of that, Mansfield admits, is due to companies locating near the best research, but even companies that are not close to the best universities find it valuable to support research in nearby, less highly rated schools.

Almost all the individual researchers were funded at least in part by the federal government. Five out of every six also received money from industry to support their research, although industry provided a much smaller average percentage of the funding than government (23 per cent against 63 per cent).

More than half of the researchers told Mansfield that the direction of their research had been influenced considerably by the funders, although two-thirds of them insisted that they relied primarily on their own judgement.

Mansfield is cautious about how valuable his studies are for making funding decisions. "It's nice to know that in the past these returns have been considerable," he says, "but these findings alone do not prove anything about the future." The main message, Lederman says, should be that academic research is a good investment.

Robert Pool

Cool and out of sight

Anthony Whitworth

THE reported discovery of "possibly the youngest stellar object yet found" by G. Sandell *et al.* (*Astrophys. J.* **376**, L17–L20; 1991) is bound to generate interest. With a temperature of around 37 K, and made of diffuse gas and dust distributed over an area much larger than the Solar System, the object hardly resembles what one generally regards as a star. Rather, it provides vital evidence of what happens as cold interstellar clouds collapse to form new stars.

The search for a 'true protostar' has been the holy grail of infrared astronomy for over twenty years (C. G. Wynn-Williams *A. Rev. Astr. Astrophys.* **20**, 587–618; 1982). All this time, this elusive beast has been lying low and keeping cool, thereby avoiding detection in the near- and mid-infrared. Only with recent advances in far-infrared and submillimetre observing techniques has it become possible to winkle out true protostars and distinguish them, with some confidence, from stars deeply embedded in clouds of dust.

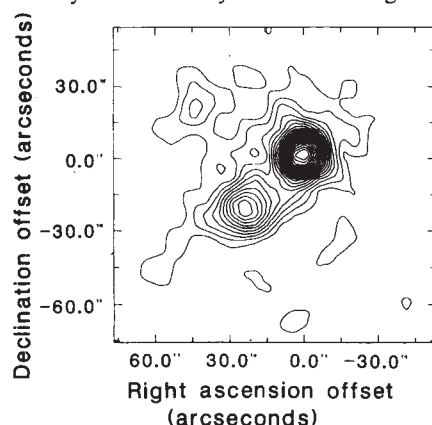
To understand why true protostars have been so hard to track down, and why they have been sought with such vigour, it is appropriate first to ask what is meant by a 'true protostar'. More precisely, as everyone would agree that a protostar is a body of gas which is irreversibly destined to become a star, we should ask at what point a protostar becomes a star. By far the commonest answer is when nuclear burning starts to supply most of the object's luminosity. But this means that all pre-'main-sequence' objects, except those burning deuterium, are protostars: and hence that the first protostar was detected many decades ago.

The next most common answer asserts that any pre-main sequence object which generates most of its luminosity internally, either by nuclear reactions or by quasistatic gravitational (or Kelvin-Helmholtz) contraction, is a star. But this means, for instance, that those T Tauri stars whose luminosity is generated mainly by accretion of material from a circumstellar disk are protostars. So again this would imply that the first protostar was detected long ago.

The true protostar that has eluded astronomers all these years should tell us something crucial about the early dynamical stages of star formation — the stages which presumably determine the efficiency of star formation, the range of stellar masses, the statistical occurrences of binary stars and the properties of young star clusters. If this viewpoint is accepted, then a true protostar becomes

a star as soon as most of its mass has adopted a configuration which is close to hydrostatic balance (as distinct from centrifugal balance) and when it has developed a photosphere.

This does not exclude the possibility that the photosphere of a formed star is heavily obscured by a surrounding co-



Far-infrared map of NGC1333 IRAS 4, perhaps the youngest stellar object discovered so far.

coon (or disk) of gas and dust. Nor does it exclude the possibility that most of the photospheric luminosity of the star is supplied by accretion of this gas and dust. But it does mean that the matter in a star has erased most of the record of its earlier history. A true protostar, in contrast, still retains detailed information about its genesis, which is why observations of a true protostar are so essential to understanding star formation.

What, then, does a true protostar look like? And how is it distinguished from a very young but deeply embedded star? Theory suggests that the condensation of a protostar can be divided into two phases. In the first stage, the protostellar gas is at a roughly uniform temperature of about 10 K, and so the condensation approaches freefall collapse. Because its temperature is so low, the protostar during this first phase is virtually dark, making it rather hard to observe the details of the collapse velocity field.

Any slight (and inevitable) rotation of the protostar will prevent, through conservation of angular momentum, the collapse from continuing in all directions. Rather, the protostar will develop into a disk. The subsequent evolution of this disk constitutes the second phase of protostellar evolution, and is controlled by the viscous stresses that transport angular momentum outwards, thereby allowing matter to spiral inwards to build up a star at its centre. The protostar also starts to heat up when it forms a disk, both because it becomes increas-

ingly opaque to its own cooling radiation, and also because the rate of compressional heating increases. Its temperature rises to 20–40 K and its luminosity increases.

At this stage the protostar should become detectable as an extended source of far-infrared continuum emission. Its spectral energy distribution (SED) should resemble that of a blackbody at 20–40 K, but with a shortfall at very long wavelengths to which it is relatively transparent. An SED with these properties is normally designated extreme class I (for example C. J. Lada in *The Physics of Star Formation and Early Stellar Evolution* (eds C. J. Lada & N. A. Kylafis) 329–363 (Kluwer, Dordrecht, 1991)).

Eventually, the protostellar disk stops growing, as infall from the earlier collapse dries up, and starts to shrink as the disk matter is either accreted onto the growing central star or blown away by a wind from the central star. It also becomes more luminous, as the protostellar material falls deeper and faster into its own gravitational potential well.

Once most of the mass is in the central star (rather than in the disk) and the luminosity is generated mainly by accretion onto the central star (rather than by accretion onto the disk and viscous dissipation in the disk), the protostar becomes a star. As this happens, the SED changes to become much broader, ceasing to resemble a single-temperature blackbody. This is because the near- and mid-infrared radiation from the central star and from the hot dust in its vicinity starts to shine through the thinning over-layers of cooler dust.

The new report by Sandell *et al.* is of a very cool low-luminosity binary system with an extreme class I SED, which they suggest is not only a true protostar, but the youngest true protostar yet identified. The authors base their identification on observations of the active star-forming region NGC1333 IRAS 4 made with the James Clerk Maxwell Telescope in Hawaii. These observations, in combination with earlier ones from the Infrared Astronomical Satellite (IRAS), allow Sandell *et al.* to construct the net spectrum of the source between wavelengths of 50 μm and 2 mm, and so to evaluate its temperature and total luminosity.

The authors also present detailed maps of the source at wavelengths of 450 and 800 μm (see figure). These show that the submillimetre emission is dominated by two components (A and B) separated by about 30 arcseconds, equivalent to a linear separation of at least 10^4 astronomical units (1 AU is the Sun–Earth separation). The two components appear to be connected, and it seems unlikely that they are unrelated,

separated objects that happen to appear on the same line of sight; so the binary identification seems reasonably secure. It may be possible to make radial velocity measurements through the spectroscopy of molecular lines, which could provide a useful constraint on the orbit and the total mass of the putative binary system.

Although the two components are comparable, component A is somewhat larger and more luminous than B, and it is on component A that Sandell *et al.* focus their attention. From its elongated image (3,200 by 2,200 AU), they suggest that it may be a disk — either a fat disk, or a thin disk seen in projection. Again radial-velocity measurements might help to clarify this suggestion. (It should be emphasized, however, that the rest of their analysis of component A does not depend strongly on whether it is a disk or not.)

The key feature of component A is the extremely low dust temperature, 37 K, which Sandell *et al.* deduce from the spectrum. In combination with its angular size, this temperature implies that component A is optically thick at a wavelength of 1.1 mm, and hence that it has an enormous column density and an exceptionally high mass, perhaps 10 solar masses. These deductions are somewhat compromised by the fact that the emissivity of interstellar dust at far-infrared wavelengths is unknown, and so there is a canonical, adjustable parameter β which enters into the evaluation of temperature and column density. The mass depends also on the detailed geometry of component A and its distance; but Sandell *et al.* claim that within these uncertainties, the mass must exceed 3 solar masses.

No near-infrared source has been detected in NGC1333 IRAS 4, so that any star-like object in component A must be embedded deep within the dust cloud and, given the low total luminosity of the component, must contain less than 2 solar masses. Hence it seems that most of the mass of component A is in the disk, or at least distributed on a scale of 1,000 AU. In other words, component A is a protostar in the sense I have defined. Moreover, its enormous mass and extent and its low luminosity suggest that this protostar is in a very early stage of its evolution.

The fact that component A appears to be in a binary system is not surprising, as most stars are born with companions. Assuming that component B is another protostar, contemporary with component A, the less extreme appearance of B could simply be due to its being less massive than A. Since components A and B define a very wide binary system, it is not impossible that the individual components will in due course break up

into close binaries, giving a hierarchical binary system.

One of the more remarkable features of NGC1333 IRAS 4 is that, despite its apparent youth, it is already associated with a bipolar outflow detected in carbon monoxide emission. This association

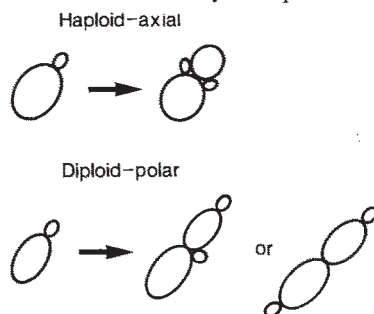
YEAST EMBRYOLOGY

Pathways of morphogenesis

Leland Hartwell

TWO reports in *Cell*^{1,2} provide a new avenue to the understanding of pattern formation in the early embryo. The work deals with control of budding in the yeast *Saccharomyces cerevisiae*, and identifies a set of gene products responsible for directing morphogenesis.

The relative sizes and positions of cells in the embryo are dictated by the positions and orientations of the planes of cell division. It is known that the division plane is determined by the position of



The different patterns of budding in haploid and diploid *S. cerevisiae*.

the mitotic spindle, but not how the movements of the spindle are programmed. It is this important process that the new observations bear upon.

The positions of individual cells in the metazoan embryo arise from a series of programmed cell divisions. Each division plane is positioned roughly midway between the mitotic poles and perpendicular to the axis of the mitotic spindle; hence the pattern of divisions reflects an underlying pattern of spindle movements. For example, in the early divisions of *Caenorhabditis elegans* the centrosomes (the poles of the spindle) rotate when necessary to position the spindle perpendicular to a specified cleavage plane, one centrosome moves towards the cortex of the cell so that the spindle is positioned asymmetrically when a division produces cells of different size³.

An elegant experiment by Breck Byers⁴ demonstrated that the site at which a bud forms on a yeast cell is also related to the positioning of the nucleus in the cell. When two haploid cells fuse the resulting zygote normally buds at the site of cell fusion, overlying the position of the newly formed diploid nucleus. When Byers displaced the zygotic nuc-

leus to one end of the cell by centrifugation he found that the site of budding was displaced to coincide with the repositioned nucleus. From cytological observations it seems that the spindle-pole body of yeast, the structural equivalent of the centrosome of animal cells, is likely to be the important nuclear element in positioning the nucleus opposite the site of budding. At the time of budding, the two spindle-pole bodies are adjacent in the nuclear envelope facing the site of budding. The spindle poles presumably communicate with the surface through the cytoskeleton: actin dots and membrane-bound vesicles are located near the site of budding and extranuclear microtubules emanate from the spindle poles toward this site.

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The positions of successive buds on a yeast cell form a pattern that is different for haploid cells (either *Mata* or *Mata α*) and diploid cells (*Mata/Mata α*) (see figure). Haploid cells bud in an axial pattern, with successive buds arising near one end of a mother cell. Diploid cells, on the other hand, bud in bipolar fashion, successive buds appearing at opposite ends of a mother cell. This difference is, however, not strictly due to differences in ploidy, but rather reflects control exerted by a mating-type locus because diploid cells that are *Mata/Mata* or *Mata α /Mata α* have the pattern characteristic of haploid cells. Because the mating-type loci are known to control the expression of a variety of genes, these observations imply that bud pattern is under genetic control.

Chant and Herskowitz¹ set out to identify genes that control bud pattern by visual examination of haploid cells subjected to mutagenesis. They identified recessive mutations in four genes, *BUD1-4*; an accompanying paper² describes a fifth gene, *BUD5*. Mutations in *BUD1*, 2 or 5 result in random positioning of the budding site in either haploid or diploid cells. Haploid or diploid cells containing mutations in *BUD3* or 4 exhibit a bipolar pattern normally characteristic of diploid cells.

To explain these phenotypes Chant and Herskowitz suggest that the default pattern for bud positioning (the pattern without genetic programming) is ran-

dom. *BUD1*, 2 and 5 convert the random pattern to a bipolar pattern; *BUD3* and 4 convert the bipolar pattern to an axial pattern. The authors propose that diploid cells exhibit the bipolar pattern because the presence of both mating-type alleles represses expression of *BUD3* and 4. Haploid cells exhibit the axial pattern because all five genes are active. The *PAR* genes of *Caenorhabditis elegans* seem to play an analogous role to the *BUD* genes of yeast — mutations in *PAR* alter the orientation and position of the mitotic spindle in the early embryonic divisions of the worm⁵.

The polarity of some divisions during embryogenesis is probably controlled by a genetic programme intrinsic to the dividing cell, but the polarity of other divisions is likely to be induced by signals from neighbouring cells. It is noteworthy, therefore, that nuclear orientation, cytoskeletal organization and localized cell-wall growth of yeast can be directed by the pheromone gradients of neighbouring cells during mating as well as by the internal genetic programme identified here. Whether any of the *BUD* genetic functions are involved in the cell's polarized response to its mating partner remains to be seen.

Although the *BUD* genes seem to dictate the position of the bud, other genes (*CDC24*, *CDC42*, *CDC43* or *BEM1*) studied in the Pringle laboratory are responsible for confining wall growth to the bud site. Mutants with defects in these genes fail to bud. The genes that specify bud position must interact in some way with those that restrict wall growth to the chosen site. Clues to the biochemical function of these genes, and their possible modes of interaction, are to be found in their DNA sequences. *BUD1* and *CDC42* are strikingly similar in sequence to the GDP-GTP exchange family of RAS-related proteins. *BUD5* shares sequence similarity with *CDC25*, a protein that promotes GDP-GTP exchange on yeast RAS⁶. In addition, *BEM1*, which localizes to the site of budding contains a potential cytoskeletal-binding domain (SH3), and is therefore a strong candidate for the agent that mediates interactions between the bud site at the cell surface and the underlying cytoskeleton (J. R. Pringle and I. Herskowitz, personal communications, and ref. 7).

The authors of these papers do not put forward an answer to the perplexing question of why a unicellular organism such as yeast would go to the trouble of programming the orientations of its divisions when it is not producing an embryo. Maybe polar budding in diploid cells promotes the dispersion of nonmotile daughter cells to better use nutrients,

DYNAMICS

Deciding the undecidable

Ian Stewart

THE central thesis of Roger Penrose's *The Emperor's New Mind*¹ is that nature produces harnessable noncomputable processes, but at the quantum level only. He goes on to speculate that human intelligence may be such a process. Two recent papers, by N. C. A. da Costa² and F. A. Doria³, cast doubt upon Penrose's thesis, and explore the limits of computability in chaotic classical dynamical systems.

The standard model of the computational process was introduced by Alan Turing: it is an abstract programmable computer, consisting of an infinite tape bearing symbols 0 or 1. The tape can be moved past a reading head which prints or erases symbols according to a fixed 'program'. Turing machines can simulate any known computational process. Turing used them to prove the existence of uncomputable functions, and also the insolubility of the 'halting problem': to decide by a fixed procedure whether a given program will terminate in a finite number of steps when executed upon a given Turing machine. In a similar vein, Kurt Gödel proved that within any formal description of arithmetic there must exist true statements that cannot be proved (incompleteness) and statements whose truth or falsity cannot be decided algorithmically (undecidability).

A huge range of questions — mostly algebraic or combinatorial — has since been proved undecidable. da Costa and Doria show that undecidability extends to many basic questions in dynamical systems theory. These include whether the dynamics is chaotic, whether a trajectory starting from a given initial point eventually passes through some specific region of phase space, and whether the equations are integrable — that is, possess an explicit solution defined by formulae built up from classical functions such as polynomials, sines and exponentials. Indeed virtually any 'interesting' question about dynamical systems is — in general — undecidable.

This does not imply that it cannot be answered for specific systems of interest: on the contrary an important recent breakthrough has been the proof by M.

whereas axial budding in haploid cells promotes the interaction of clonal progeny for mating. Alternatively, perhaps evolution foresaw the need of geneticists to have a truly universal cell. □

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Benedicks and L. Carleson⁴ that the Hénon system (the classic example of nonlinear dynamics) possesses chaotic attractors. However, it does demonstrate the absence of any general formal technique. Other recent 'uncomputability' results in dynamics include C. Moore's example⁵, discussed in these columns by C. Bennett⁶, of a deterministic system with uncomputable trajectories; and the theorem⁷ that Julia sets — and, assuming one plausible conjecture, the celebrated Mandelbrot set itself — are uncomputable. That this may be so, despite the walls across our planet being papered with computer pictures of Julia and Mandelbrot sets (see figure), hinges upon the infinite precision required in the theoretical computations.

A heuristic approach indicates the possibility of curious computational-type behaviour in classical dynamical systems. It is clearly possible to represent the behaviour of an electronic computer — or more simply a genuinely mechanical model of a Turing machine — as a dynamical system: one merely writes down idealized but realistic equations for its electronic components. Because classical mechanics poses no upper bound upon velocities, it is possible to accelerate time in these equations, so that infinite real time passes within a finite period of fake time. Thus we have a system of classical equations which correspond to an infinitely fast computer. (Imagine a Turing machine with a tape that accelerates so rapidly that it can complete an infinite number of operations in one second.) Such a machine can in principle compute functions which, in the usual sense of mathematical logic, are uncomputable. For example, it can solve the halting problem by running a computation for infinite real time and throwing a particular switch if and only if the program stops. In fake time this entire procedure is over within a fixed period: then one merely inspects the switch to see whether it has been thrown. Indeed such a machine could prove or disprove Fermat's last theorem (the equation $x^n + y^n = z^n$ has no nonzero solutions in whole numbers when $n \geq 3$)

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RÉSUMÉ

In the gap

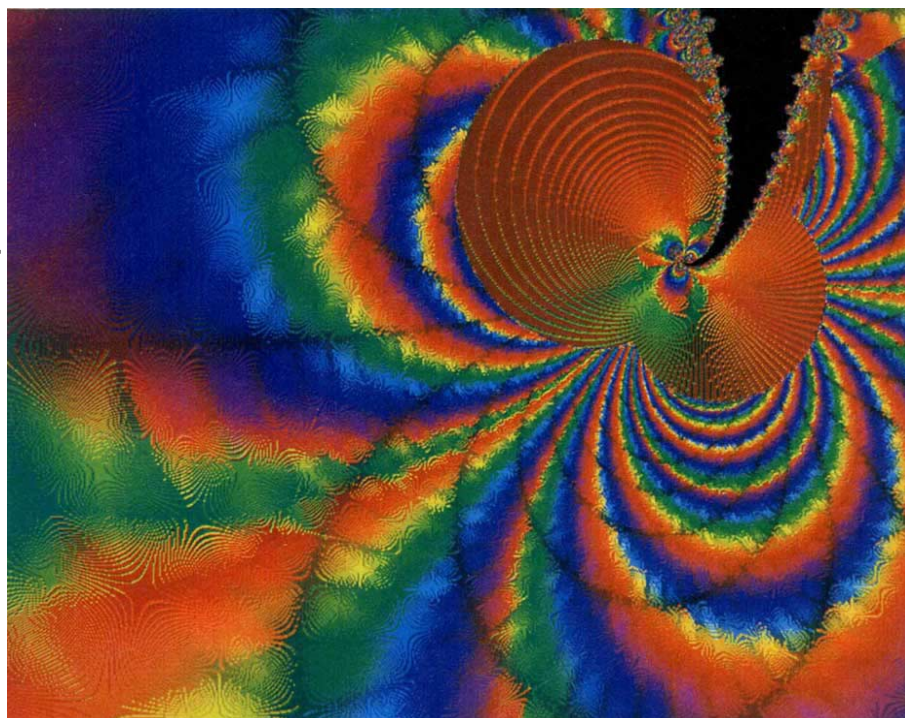
DELETIONS of a portion of the long arm of chromosome 15 are associated with two phenotypically distinct disorders, Prader–Willi syndrome and Angelman syndrome; the difference depends on which parental chromosome suffers the deletion, and provides strong evidence for genetic imprinting. J. Wagstaff *et al.* (*Am. Soc. hum. Genet.* **49**, 330–337; 1991) now report the first known gene to map to this region (15q11–13), that encoding the $\beta 3$ subunit of the GABA_A (γ -aminobutyric acid) receptor. Although one copy of the gene is commonly deleted in people suffering from the syndromes, both copies were present in one Prader–Willi patient with an unbalanced chromosomal translocation. Nevertheless, the possible involvement of this neurotransmitter receptor in the pathogenesis of the two disorders will no doubt now be the subject of detailed scrutiny.

Binges and bagels

PEOPLE like to eat, and they like it more when they do it with other people. The more the merrier, says J. M. De Castro in *Physiology & Behaviour* (**49**, 1289–1291; 1991), who paid 315 people \$30 each to keep 7-day diaries recording everything they ate, where and when they ate it, and how many other people kept them company. The correlation between meal size and company remained rock-solid even when weekend binges and drunken excesses on a Saturday night were taken into account. Social facilitation, claims De Castro, could be the most important determinant of normal human eating behaviour. The results corroborate the old story of the rabbi at a social function who was offered another bagel. “No, I couldn’t possibly” he says, “I’ve already had six”. “You’ve had seven”, came the reply, “but who’s counting?”.

Seeing red

G. ALBRECHT-BUEHLER (*J. Cell Biol.* **114**, 493–502; 1991), asking whether cultured cells will respond to infrared light, has received an affirmative answer. When tickled with a few quanta, 3T3 cells it seems (some of them at least) extend pseudopodia towards the light source. The preferred wavelength is 800–900 nm and pulsed irradiation generates the most activity. The heating effect is negligible so the cells must have a chromophoric receptor, a pigment presumably (though it ought to have absorption bands at shorter wavelengths also). And how does this curious faculty profit the cells? Albrecht-Buehler allows himself some fairly uninhibited speculations: perhaps infrared intensity is attenuated by a chemotactic concentration gradient, which the cells might then more easily sense, or perhaps the cell is an emitter as well as detector of radiation and exchanges signals with its friends. But could the process simply help them to avoid bumping into things in the dark?



The art of the impossible: a computer-generated picture of the uncomputable Julia set.

by checking all possible sums (infinitely many) of two n th powers. More ambitiously, it could prove all possible theorems in a finite period of time, by pursuing all logically valid chains of deduction from the axioms of set theory. It is clear from this that some ‘classical’ dynamical systems can simulate bizarre computations. The reason is that perfectly respectable equations have a natural interpretation as models of physically impossible devices.

However, it is far from trivial to implement this informal project in a logically impeccable manner. For example, one must actually write down the equations — or at least indicate how this could be done. And one must deal with the potentially infinite-dimensional space of positions on the Turing machine’s tape. da Costa and Doria use a quite different approach. Their basic idea is inspired by a remark of B. Scarpellini⁸ that it may be possible “to build an analog computer that can simulate functions $f(x)$ so that the predicate $[\phi(f(x))]$ isn’t decidable, while the analog machine itself decides it”. Developing this idea, da Costa and Doria construct a series of functions $f_m(x)$, obtained from classical functions and integrals that involve them. These functions are either identically 0 or identically 1, but their value is formally undecidable.

da Costa and Doria define a family of classical (that is, not quantum) dynamical systems, one for each m , each being either a free particle or a simple harmonic oscillator, depending upon the value of f_m . Since the value of f_m is undecidable, there is no formal way to compute

which is the case. However, one can imagine a thought experiment in which an analog device simulates the dynamics for any given m . It will then be apparent to an observer whether or not the motion is simple harmonic or free (this choice is Scarpellini’s predicate ϕ) — in one case the particle oscillates along an interval, in the other it whizzes off to infinity — so the analog machine can effectively decide the undecidable. In this thought experiment, a classical system computes something that is algorithmically undecidable, contrary to Penrose’s thesis.

The thrust of Penrose’s thesis is thus directed elsewhere: if it is to be valid, then it must be posed for real classical systems rather than mathematical ones, a concept that is hard to handle rigorously in a world that is really quantum. Clearly some rethinking is in order here. Dynamical systems theorists may object that the da Costa–Doria family is artificial and thus has no implications for systems that actually arise in applications. It is worth remembering that similar objections were raised to the work of Turing and Gödel. □

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Virus receptors as permeases

Richard G. Vile and Robin A. Weiss

KIM *et al.* and Wang *et al.*, on pages 725 and 729 of this issue^{1,2}, and B. O'Hara at a recent Cold Spring Harbor Laboratory conference, report that two retroviruses use cell-membrane permease proteins to gain entry to their target cells. These findings are a landmark in mammalian cell physiology, open a new chapter in research into viral receptors and, because the retroviruses concerned cause leukaemia, may yet reveal new mechanisms of viral pathogenesis.

Viruses exploit cellular surface molecules as receptors for binding and entry into host cells. Two approaches have been particularly productive in identifying such receptors — antibodies to cell-surface antigens have been used to block viral attachment or infectivity, and DNA transfer has been used to recover genes which confer infectivity by the virus to otherwise non-permissive cells. Thus CD4 was first identified as the receptor for human immunodeficiency viruses by screening antibodies to leukocyte antigens and, later, when the complementary DNA for CD4 was cloned, by DNA transfer. Likewise, further receptors for mammalian viruses have been identified as cell-surface antigens, for example CR2 for Epstein-Barr virus and ICAM-1 for rhinoviruses. In other cases, DNA transfection has brought to light such previously unrecognized molecules as the receptor for poliovirus and those for two retroviruses, ecotropic murine leukaemia virus (MuLV-E)³ and gibbon ape leukaemia virus (GALV)⁴.

The gene for the receptor for MuLV-E (ecoR) encodes a protein of 622 amino acids with 14 potential membrane-spanning domains³. Although the protein is not similar in sequence to other known proteins, the transmembrane topology is reminiscent of that of several other membrane transporter proteins, most notably the permeases for arginine, histidine and choline of yeast. Following up this clue, Cunningham's¹ and Kabat's² groups searched for a similar transporter function of ecoR.

Both groups now demonstrate that *Xenopus* oocytes injected with ecoR messenger RNA show increased uptake of the basic amino acids L-lysine, L-ornithine and L-arginine. Only in the presence of extracellular sodium were the injected oocytes capable also of transporting the neutral amino acid homoserine. This sodium-independent transport of cationic amino acids closely resembles the properties of a physiologically well-characterized amino-acid transport pathway of mammalian cells, known as y^+ (refs 5 and 6). From the

functional data, the largely ubiquitous tissue co-expression of y^+ and ecoR, and the structural similarity to yeast amino-acid transporters, it seems certain that ecoR and y^+ are one and the same, making this the first mammalian amino-acid transporter to be cloned and the first instance of a virus subverting a transmembrane channel protein as a receptor.

Not only will this revelation open up the field of mammalian amino-acid transport physiology, but also, as Wang and colleagues suggest, it may lead to the definition of inherited genetic diseases caused by abnormal cellular trans-

port of amino acids (just as the defect in cystic fibrosis is caused by mutations to a putative chloride transporter). Cell physiologists may also be able to benefit from the work already performed on the pathways of viral entry following binding — the methods by which virus-receptor complexes are recruited into coated pits (receptor-mediated endocytosis) or fuse to the cell membrane after binding should help in unravelling the normal processes involved in turnover of these transporter proteins at the cell surface^{7,8}.

Last year, a cDNA that confers susceptibility to infection by GALV was cloned, the predicted protein sequence being 679 amino acids in length, containing membrane-spanning domains and having no detectable sequence similarity to other known proteins⁴. Now, O'Hara and colleagues (Lederle laboratories,

Snap, crackle and pop

IMAGE
UNAVAILABLE
FOR COPYRIGHT
REASONS

Gordon Tribble

LAVA from the slopes of Kilauea volcano on Hawaii island can flow as far as the sea. Gordon Tribble of the US Geological Survey and colleagues went scuba diving in 1989 to find out its fate. At the time, lava had been flowing continuously from the eastern flanks of the volcano for two years. It reached the coastal edge 12 kilometres away through shallow tubes encased within the older, cooled lava, making hot fire spouts that drained straight into the sea. The divers' efforts were rewarded by the observation of a new type of lava flow, streams about a metre wide that advanced at a speed up to 3 metres a second down the steep submarine slopes. Although they could not trace the lava so far, the researchers suggest the streams could plunge to depths of 100 metres or more. At times, Tribble's description of the dives (*Geology* **19**, 633–636; 1991) reads like a boys-own adventure. One colleague was buried up to his knees as rocks and debris slumped down the steep submarine slope. "Frequent loud popping sounds were superimposed on a constant background noise of sizzling and cracking" Tribble records. "Resonant booms and concussions punctuated the activity." Much of this noise Tribble associates with the occurrence of fissures in the cool crust overlaying the streams, like that shown here, in which water became heated. The gas bubbles that emerged from the lava (some looking like 30-centimetre smoke rings) contained not only steam, but also hydrogen and oxygen from thermally dissociated water, which could spontaneously combust. □

personal communication) have found that this human gene is very similar in sequence to a gene of the fungus *Neurospora crassa* which encodes a phosphate transporter protein. It will be interesting to determine whether *Tea*, a gene which has some sequence similarity to that for *ecoR*, and which is expressed in activated T cells and maps in the same region as the gene for the amphotropic MuLV (MuLV-A) receptor⁹, also encodes a permease.

Of what value are these findings to virologists, particularly in the understanding of leukaemia retroviruses? It has often been proposed that virus binding may initiate intracellular signals that set off a chain of events leading to disease, and there have been reports of virus binding to cells leading to specific gene, and cell, activation events for both retro- and other viruses¹⁰⁻¹². But it remains to be seen if the subversion of permease proteins by MuLV-E and GALV has any analogous relevance to the leukaemogenic process. The addition of purified viral glycoprotein (gp70) or virus particles to oocytes injected with *ecoR* did not alter the amino-acid transport properties of the protein, even though the oocytes specifically bound gp70 (ref. 2). Therefore, the binding of virus does not seem to disrupt the transport properties of the receptor; indeed, it has long been known that cells persistently infected with MuLV-E, in which gp70 is expressed, are viable and grow well. It is likely that the site of viral adsorption will be separate from the domain of metabolite transport, otherwise virus binding and surface envelope expression would lead to blockage of the essential transport function and to cell death rather than indefinite cell growth (transformation).

There is also much evidence to implicate post-penetration events in the pathogenesis of cellular infection by the leukaemia retroviruses, particularly proviral insertional mutagenesis for MuLV-E and gene transactivation by the human T-cell leukaemia viruses types I and II. So selection of cellular permease pro-

teins as receptors may reflect an advantageous 'choice' for the virus simply because this class of protein, involved as it is in the transport of essential metabolites, is expressed on a wide variety of cell types.

Interestingly, the receptors for three distinct human viruses — HIV, poliovirus and rhinovirus — belong to the immunoglobulin superfamily. We do not know whether this is fortuitous, or whether the normal function of these receptors relates to their adoption during viral evolution. The identification of amino-acid and phosphate permeases as

CELL PHYSIOLOGY

A sense of cell size

Andrew R. Cossins

THREE new reports¹⁻³ make clearer the mechanisms by which cells control their volume — a process which is essential for normal cellular function. Such control has to be exercised in the face of substantial solute fluxes, a vigorous metabolism and the Donnan effect, and in part is achieved by regulation of the net movement of specific ions and amino acids down their concentration gradients as a means of moving osmotically obliged water⁴. Yet despite the obvious importance of transporter regulation, progress in identifying the transducer mechanisms linking cell volume to transporter activity has been disappointingly slow. Two studies on mammalian red cells^{1,2} and one on duck red cells³ now firmly implicate the phosphorylation state of key proteins in the control of two volume-regulatory transport systems.

Ideas on the control of volume-sensitive transport mechanisms fall into two camps. The first is that a mechanical strain sensor in the cell membrane or its associated cytoskeleton modulates the transporter^{5,6}; stretch-activated conductive channels have been identified in various cell types and they may form the transducer/transporter element in certain cells, including T lymphocytes⁷. The second is that cell swelling alters the intracellular concentration of some aqueous moiety and that this signal initiates events that lead to transporter activation⁸. This mechanism requires a means of amplification because a small change in cell volume may lead to disproportionately large increases in permeability. Distinguishing between the two models has not proved easy, especially as many volume-sensitive transport systems involve electroneutral exchanges of ions and are not amenable to patch clamp analysis.

The red cells of many vertebrate species possess a K flux mechanism that is

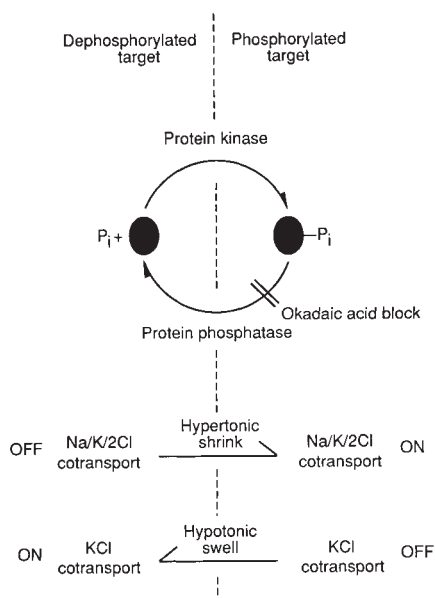
activated by cell swelling and inhibited by cell shrinkage. The mechanism depends on the presence of Cl and probably represents an electroneutral cotransport of KCl. Jennings and coworkers^{8,9} have proposed that, in rabbit red cells, these transporters exist in one of two states, active and resting. They have also shown that a simple model involving a cycle of two enzymes which activate and inhibit the transporter, and which are differentially affected by dilution, can account for volume sensitivity.

Now Jennings and Schulz¹ find that okadaic acid, a toxic polyether fatty acid which was first isolated from a marine sponge and is responsible for diarrhetic food poisoning, completely inhibits the swelling-activated KCl flux mechanism, and Kaji and Tsukitani² observe an identical effect in human red cells. Okadaic acid is reported to inhibit phosphatase types 1 and 2A only¹⁰, and this specificity is the basis for invoking the involvement of protein phosphorylation state in the control of the KCl cotransporter.

Avian red cells are different, in that they display a Na/K/2Cl cotransport mechanism rather than the KCl cotransport of mammalian red cells. Moreover the Na/K/2Cl system is activated by cell shrinkage rather than by cell swelling, and is also sensitive to other stimuli including agents which raise the levels of intracellular cyclic AMP. Pewitt and colleagues³ show that protein kinase inhibitors inactivate cAMP-induced cotransport, presumably by blocking the cAMP-dependent protein kinase. More importantly, they demonstrate that okadaic acid causes its activation even in the absence of any of the normal stimuli such as cAMP or cell shrinkage.

The idea is that blockage of phosphatase activity leads to the progressive phosphorylation of some regulatory component by kinase activity, and that

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The phosphorylation cycle and the corresponding effects of cell swelling and shrinkage upon activity of the volume-regulatory cotransport systems in human and duck red cells.

this switches on the transporter (see figure). In quiescent cells phosphatase activity dominates, the fractional phosphorylation state of the protein is low and the transporter is switched off. Stimulation by cell shrinkage involves a change in the rates of phosphorylation relative to dephosphorylation so that fractional phosphorylation increases and the transporter is progressively activated. Pewitt *et al.*³ have identified a glycoprotein of relative molecular mass 152,000 which specifically binds bumetamide, a high-affinity inhibitor of transport, and which is also phosphorylated. They speculate that this may be the phosphorylated transporter itself.

At first sight, the transduction mechanisms of these two cotransport systems appear to have little in common because one is inhibited by okadaic acid (human) whereas the other is activated (duck). But similarities emerge when the effect of changes in cell volume upon phosphorylation is incorporated into the picture. Hypertonic shrinkage in duck red cells causes the activation of the Na/K/2Cl system by an increase in the fractional phosphorylation of some, as

yet unidentified, protein. By contrast, cell swelling in human and rabbit red cells causes the activation of the KCl system by a decrease in phosphorylation. Or looked at another way, cell shrinkage inactivates the cotransporter by an increase in phosphorylation.

So, in both types of red cell, variations in cell volume have consistent effects upon the phosphorylation of some essential component(s) of their respective transduction mechanisms. The result is an adjustment of the activity of the

OZONE DEPLETION

A deepening, broadening trend

Guy P. Brasseur

SINCE late 1978, atmospheric ozone has been continuously monitored by the Total Ozone Mapping Spectrometer (TOMS) on the Nimbus 7 satellite. A revised version of the data, corrected to ensure internal consistency between the ozone values derived from measurements at different wavelengths¹, has now been used by Stolarski *et al.*² to derive the trends in atmospheric ozone over a 11½-year period up to May 1990. Their study shows that between 65° N and 65° S, the ozone column abundance has decreased on the average by 3 per cent over the whole period. But the trend is not geographically uniform. Although the variation is extremely small (and not significant) near the Equator (20° N to 20° S), statistically significant depletion becomes apparent in both hemispheres polewards of 30°. The reduction in the ozone column is particularly large (3 per cent per year on the average) near the Antarctic continent especially in spring and, even at 60° S, a mean trend of -0.6 per cent per year is reported. These large perturbations are clearly associated with the formation of the Antarctic ozone hole. An important yet poorly understood conundrum is how the ozone-poor air from the polar region is mixed into lower latitudes in late spring (November) when the polar vortex (the home of the hole) breaks down.

In the Northern Hemisphere, the largest depletion appears to have taken place at mid-latitudes. Stolarski *et al.* report an average depletion of 0.4–0.5 per cent per year in the belt 40–50° N with trends of -0.2 per cent per year in summer, and -0.8 per cent per year in winter. If these numbers are correct, the yearly integral of the ultraviolet-dose in the spectral region where DNA is easily damaged should have increased by about 10 per cent over the past decade, with wintertime increases of about 20 per cent (S. Madronich, personal communication). Models simulating only gas-phase, homogeneous reactions in the stratos-

phere do not account for such large ozone reduction in the northern mid-latitudes and other explanations must therefore be found.

It should be noted that the trends presented by Stolarski *et al.* are substantially stronger than those³ based on the analysis of data gathered between 1970 and 1986 by the global network of ground-based 'Dobson' spectrophotometers. This acceleration with time in the ozone depletion could reflect the existence of nonlinear processes such as those involving heterogeneous reactions on droplets and ice particles⁴. A direct influence of the high-latitude regions, where polar stratospheric clouds are observed during winter, is therefore plausible. Observations made in the Arctic region during the Airborne Arctic Stratosphere Experiment⁵ in 1989 show that concentrations of active chlorine are comparable with those observed in the Antarctic stratosphere during spring, when ozone is greatly reduced over a period of 2–3 weeks. The dynamics of the stratosphere in the Northern Hemisphere is significantly perturbed by large-scale planetary waves, generated by airflow over the Rockies and Himalayas, so that air masses processed inside the Arctic stratospheric clouds are likely to be transported to mid-latitudes where solar radiation is available to drive the photochemical destruction of ozone.

Another possible explanation involves the combined action of sulphate aerosols and nitrogen and chlorine chemistry. Laboratory work⁶ suggests that heterogeneous reactions similar to those observed on ice crystals inside polar stratospheric clouds could also take place on the small H₂SO₄/H₂O droplets forming the so-called Junge layer in the lower stratosphere (14–25 km). Calculated ozone trends at mid- and high latitudes are closer to the observed trends, when such heterogeneous reactions are taken into account⁷. The surface area available for these reactions is

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substantially smaller than in the polar stratospheric clouds, but, especially in the years following a large volcanic eruption such as that of El Chichon in 1982, the effect could be noticeable^{8,9}. The efficiency of these processes depends on the chlorine load of the atmosphere, and so is gradually increasing as a result of the use of chlorofluorocarbons. If the amount of sulphur injected into the stratosphere by the eruption of Mount Pinatubo in the Philippines in June is 2 or 3 times larger than that emitted by El Chichon, as is indicated by satellite observations, a reduction in the ozone column could, according to our model calculations, reach significant values during next winter at mid- and high latitudes, after the volcanic material has spread out over the entire Northern Hemisphere.

It has also been suggested¹⁰ that substantial amounts of ozone could have been destroyed as a result of increased production of nitrogen oxides by energetic protons in the solar wind following the large solar flares of 1989. Nitrogen oxides are known to destroy ozone catalytically at altitudes of 20–50 km. Large reductions in ozone concentrations in the upper stratosphere were reported¹¹ after a similar event in August 1972. Although TOMS data seem to indicate that a limited amount of ozone was destroyed in the vicinity of the South Pole¹⁰ following the two proton events in March 1989, model calculations¹² show that the largest percentage reduction in the ozone concentration (about 20 per cent) following the intense flares of the second half of 1989 should have taken place near 40 km, leading to a reduction in the ozone column much smaller than the one derived by the recent trend analysis.

Evidently, the indications are that ozone in the middle atmosphere is more sensitive to anthropogenic chlorine than previously predicted by numerical models. Heterogeneous chemistry on cloud and aerosol particles both at high and

mid-latitudes is probably playing a key role that is not yet fully understood. The importance of dilution processes from the polar regions in both hemispheres also needs to be evaluated. The Arctic campaign organized by European, American and Soviet scientific groups this coming winter will provide vital information to address these questions.

NEUROSCIENCE

Back together again

Terrence J. Sejnowski

IN the 1960s, recordings from single neurons opened a new view of the visual system¹. The surprise was that single neurons carried so much information about features in the image; the eventual disappointment was that recordings from dozens of visual areas of cerebral cortex did not lead to a deeper view of visual perception. As it was put at a recent meeting*, poking around in the brain for

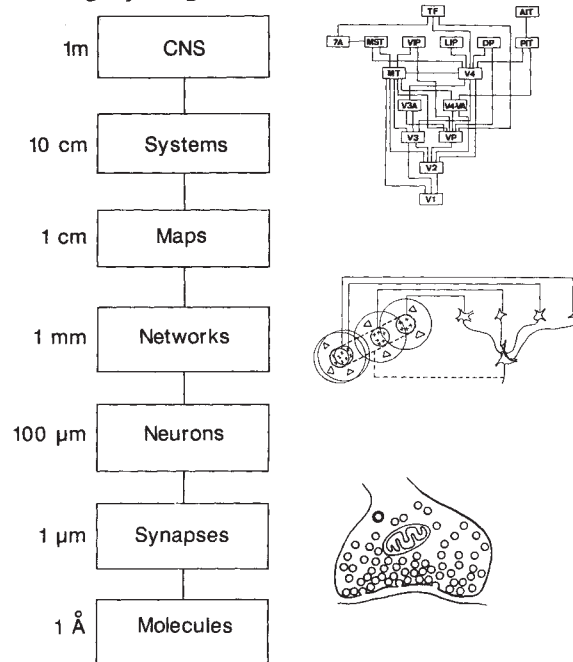
But the polar aspects of the mission might be somewhat overshadowed by the chemical and radiative perturbations associated with the recent eruption of Mount Pinatubo. □

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are changing our view of vision.

New techniques from molecular neuroscience are beginning to give us a glimpse of how the visual system is put together. Many morphologically similar neurons differentially express surface markers. It was a great surprise, however, to find that a monoclonal antibody selectively stains the precise subsets of cells in the lateral geniculate nucleus and cortical areas V1, V2, MT and MST that are part of the visual 'motion' processing system (S. Huckfield, Yale University). Visual experience is needed for normal properties of this system to develop, and also for normal expression of the membrane marker. It will be exciting to learn what the function of the molecule might be, and whether other functional systems can be revealed by this approach.

Recording from one cell at a time is like examining a tapestry one thread at a time. A stunning hardware simulation of the salamander retina, based on physiological recordings from horizontal, bipolar and amacrine cells (F. Werblin, University of California, Berkeley), shows the response of a large array of neurons and is like seeing an entire tapestry for the first time. The model of how can be used to gain insights into the consequences of changing key parameters, such as electrical coupling between horizontal cells, and to develop a better sense for how motion is represented in the array of ganglion cells⁵. A wide range of modelling and theoretical studies were presented — from detailed models of cone adaptation (D. Tranchina, New York University) to network models of disparity selectivity in visual cortex (R. Freeman, University of California, Berkeley). R. Barlow has programmed a parallel computer called the



Levels of organization in the nervous system. The spatial scales (left) at which anatomical organizations can be identified varies over many orders of magnitude. Icons (right) represent structures at district levels: (top) a subset of visual areas in visual cortex; (middle) a model of how can be used to gain insights into the consequences of changing key parameters, such as electrical coupling between horizontal cells, and to develop a better sense for how motion is represented in the array of ganglion cells⁵. A wide range of modelling and theoretical studies were presented — from detailed models of cone adaptation (D. Tranchina, New York University) to network models of disparity selectivity in visual cortex (R. Freeman, University of California, Berkeley). R. Barlow has programmed a parallel computer called the

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Connection Machine to model the lateral eye of *Limulus polyphemus* and may, at this moment, be making video recordings in the Atlantic Ocean to use as visual input for his model.

The retina is able to adapt to complex temporal as well as spatial patterns of light (T. Reuter, University of Helsinki). If an arbitrary light stimulus is repeated at constant intervals, most ganglion cells of the frog retina cease to produce a strong response. This is due to neither light adaptation nor spike adaptation, for slightly changing the frequency immediately restores the full response. R. Desimone (NIMH, Bethesda) presented evidence that many neurons in inferior temporal cortex of macaques adapt to complex visual stimuli after a single exposure. If each stage of visual processing adapts out those aspects of the stimuli that it has analysed, only unexplained information reaches higher stages, as H. Barlow (University of Cambridge) has suggested. Knowing the right question to ask is often the key to progress, and Barlow provoked the audience by asking: "Why do you need your cerebral cortex?". At Oxford it was agreed that this was a very good question, but the most memorable answer was suggested to him by a Cambridge student: "You need your cerebral cortex so you know when to shut up". The answer may depend upon the species — in rats, the cortex is not needed for the acquisition of conditioned fear, but is required to extinguish this response (J. LeDoux, New York University).

The output of the cerebral cortex is a good place to look for its behavioural significance, and eye movements are a favourable assay for visual function. S. Lisberger (University of California, San Francisco) and J. A. Movshon (New York University) have shown that the eye can track some moving targets — coloured ones in particular — that are invisible to the classical 'colour-blind' motion pathway. Moreover the tracking system receives accurate signals about the speed of these targets even when the perceptual system incorrectly registers them as moving slowly. Neurons in the foveal region of the superior colliculus, an area of the midbrain known to be important for saccadic eye movements, have been discovered that discharge during active fixation of a visual target (R. Wurtz, NIMH); stimulation of these neurons delays saccades and lesions lead to an inability to suppress saccades, which occur with less than half the latency of normal saccades.

One way to go beyond the single neuron is to record from neural populations using voltage-sensitive dyes and intrinsic optical signals, and optical recordings have been used to map the spatial organization of neurons with

similar properties, such as colour selectivity and disparity sensitivity, in area V2 of macaque visual cortex (D. Ts'o, Rockefeller University). Using the same techniques, A. Grinvald (Weizmann Institute, Israel) has made movies of cortical activity — probably subthreshold signals in dendrites — that resemble the sloshing of water in a swimming pool during an earthquake.

Widely separated pairs of neurons in the primary visual cortex of cats sometimes fire together at around 30–60 Hz (C. Gray, Salk Institute), and such synchrony has also been observed in other areas of cerebral cortex (M. Abeles, Hebrew University, Israel). These correlations are relatively rare, but a distributed population of coherently firing neurons could have much greater effect on target neurons because of temporal summation⁶. It has been difficult to relate these observations to visual function, but they may teach us something about cortical circuitry⁷; for example models of inhibitory interactions between basket cells and cortical pyramidal neurons show that these inhibitory interneurons could help to synchronize local populations of neurons, and that under some conditions strong inhibition can actually speed up pyramidal neurons⁸.

Cognition is the holy grail of neuroscience. Visual attention is a window into our cognitive lives, a window that develops rapidly in infants, and it provides tags for objects as useful as any visual feature. Our short-term working memory gives us the ability to make plans and carry out complex actions, such as sequences of eye movements. Long-term memory allows us to form new habits and create new representations of the visual world. These connections between vision and behaviour were described by several speakers, and they point towards the emergence of a new world order without the traditional separation between vision, memory and motor systems. Having taken the brain to pieces, neuroscientists are now beginning the formidable task of putting it back together again. □

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Electrons on tap

HIGH-temperature superconductors are already beginning to escape into technology. The electricity distribution industry would give them a particular welcome. Unfortunately, the problems of making, laying and joining up miles of brittle monolithic ceramic cable seem insuperable. Daedalus now has another approach.

He has been inspired by a recent report that an electron beam will thread its way safely through even a winding tunnel in a superconductor, by magnetic self-repulsion from the superconducting walls. An electron beam coasting in a vacuum is, of course, as lossless as the most superconducting cable. So Daedalus now plans to distribute electricity round the country in the form of electron beams in superconducting pipes.

The great advantage is that monolithic, electrically continuous superconductor is not needed. Lengths of superconducting pipe can simply be cemented together like traditional ceramic drain pipes or sewer conduits. The joints will have to be vacuum tight, of course, and the whole thing will need to be cooled in liquid nitrogen. Repelled from the walls of the pipe, the electron beam will then travel safely down the middle, undisturbed by the many joins and discontinuities. The pipe can confidently be laid round a curve: the beam will follow it. It can even be subdivided by T-junctions and small off-takes, and controlled by standard taps and valves with superconducting gates or diaphragms. Electricity could be distributed in the same way as gas or water.

To cool a national distribution network in liquid nitrogen will call for a lot of new technology, but nothing that has not been thought about or tried already on a small scale. In a sense, electrical insulation will merely be replaced by thermal insulation. For a superconducting pipe repels electrons so perfectly that it never accumulates any charge itself. It can safely remain at earth potential.

DREADCO applications engineers are already wondering how best to use the electron beam when it arrives at the customer. The obvious approach, continuing this scale-up of vacuum-tube technology, is to let it hit a metal anode and be converted into a normal current. But electric light could be provided directly, by letting the beam hit a suitable phosphor, and television by scanning the beam over a screen. An electron-impact cooker could control the temperature-distribution of a cake or roast with a precision unmatched by any microwave oven, while a simple electron leak could give a negative-ion generator for clearing the air and inducing a cheerful mental atmosphere.

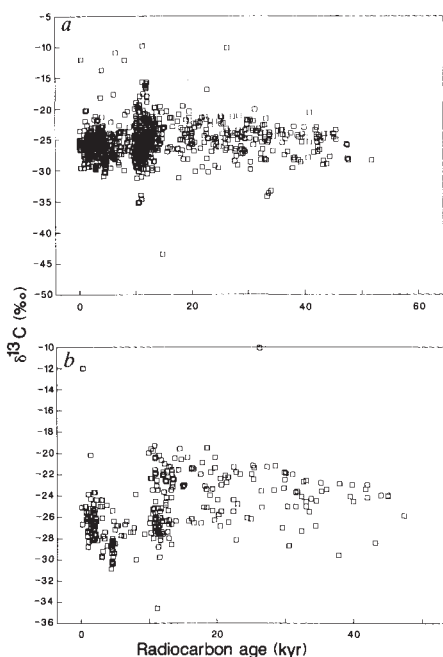
David Jones

Chronology from plant matter

SIR — In the absence of direct, long-term measurements of carbon isotope ($^{13}\text{C}/^{12}\text{C}$) ratios of CO_2 in ice cores, Krishnamurthy and Epstein have derived¹ a possible atmospheric $^{13}\text{C}/^{12}\text{C}$ chronology using isotope measurements from 115 old wood samples. They found a $\delta^{13}\text{C}$ peak at about 20 kyr before present (BP) with a decline of about 4‰ toward present and about 3‰ back to 40 kyr BP. We have tabulated more than 800 $\delta^{13}\text{C}$ values on ^{14}C -dated plant matter published in *Radiocarbon* (University of Arizona, Tucson) between 1959 and 1989. The material includes wood, charcoal, peat, leaves, twigs, pine cones and seeds.

The figure depicts the results for all data and for wood. We judged the most visible dislocation in $\delta^{13}\text{C}$ to occur at about 10 kyr BP and calculated means before and after that time. For all data, the pre-10 kyr BP mean is 0.8‰ heavier (more positive) than post-10 kyr BP ($n=821$, $P<0.01$), and for the wood the pre-10 kyr BP mean is 3.0‰ heavier than post-10 kyr BP ($n=306$, $P<0.001$). Specific subcategories of *Pinus* and gymnosperms also show a similar significant decline as that found for all wood.

The magnitudes of the wood $\delta^{13}\text{C}$ decline toward the present is comparable



a, The $\delta^{13}\text{C}$ values of all tabulated plant matter; pre-10 Kyr BP mean, -25.0‰ , s.d., 3.2‰ ; post-10 Kyr BP mean, -25.8‰ , s.d., 2.8‰ . **b**, $\delta^{13}\text{C}$ Values of all tabulated wood values; pre-10 Kyr BP mean, -24.1‰ , s.d., 2.7‰ ; post-10 Kyr BP mean, -27.1‰ , s.d., 2.4‰ . The outliers (-12 and -10‰) are either woody C4 plants or printing errors. Although included in the calculation of the means, their influence is minimal.

to that found in ref. 1, but there is no $\delta^{13}\text{C}$ peak at 20 kyr BP in the data we have analysed. Whereas Krishnamurthy and Epstein attribute the change they found to large $\delta^{13}\text{C}$ changes of atmospheric CO_2 , an increase in the average ratio of plant intercellular CO_2 to external (atmospheric) CO_2 partial pressure (p_i/p_a) from 0.78 in the glacial to 0.87 in the post-glacial alone could explain the $\delta^{13}\text{C}$ change presented here, according to plant carbon-isotope fractionation models². Isotopically heavier $\delta^{13}\text{C}$ values found in high elevation plants throughout the world³ may be evidence of lower atmospheric CO_2 partial pressure favouring reduced p_i/p_a .

Many questions remain, such as why neither our peat ($n=146$) nor our

Sperm competition

SIR — Since Parker¹ first pointed out the importance of competition between sperm from different males for fertilization of eggs of a single female, many studies have shown the importance of mating order in determining paternity patterns. Yet the underlying mechanism is known in only a few cases². In the acarid mite (*Caloglyphus berlesei*), the sperm from the last male to mate with the female has precedence over previously deposited sperm³. We have now shown that this occurs because the last male to mate repositions the sperm from previous matings, thus achieving most of the fertilizations.

The design of our experiments was based on data suggesting that no sperm are transferred in the first few minutes of copulation, but that some process which decreases fecundity of previously mated females takes place (J.R., unpublished observations). Females were allowed to mate with virgin males under three different regimes. In the control group, each female mated with one male and finished copulation naturally. In experimental group 1, naturally terminated copulation was immediately followed by matings with four other males, and each of these copulations was experimentally interrupted after 5 min. The order was reversed in group 2: four matings interrupted after 5 min were followed by a naturally terminated copulation. After matings, some females were collected to be prepared for both light and electron microscopy.

Females from group 1 deposited many fewer eggs than the control females (see table). After serial sectioning of the control females' spermathecae, a mass of several hundred sperm cells was consistently found in the basal part of the spermatheca, where efferent ducts lead-

charcoal ($n=256$) samples show any significant changes. Furthermore, analysis of material from many locations by different laboratories, in addition to inherent physiological complexities, may confound attempts at accurate reconstruction. Long reconstruction with wood or with C4 plants⁴ from a single location may offer the best hope for atmospheric reconstructions from plant matter.

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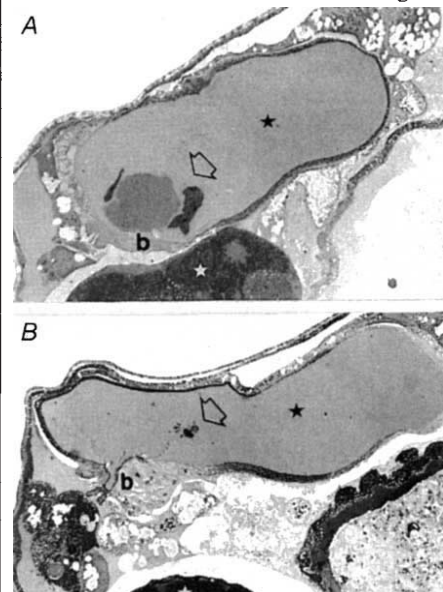
NUMBER OF EGGS DEPOSITED BY FEMALES

	Eggs per female ($\pm$ S.D.)	<i>P</i>
Control ($n=14$)	119.4 ($\pm$ 49.3)	
Group 1 ($n=8$)	25.4 ($\pm$ 25.0)	0.001
Group 2 ($n=7$)	106.6 ($\pm$ 65.4)	0.837

Eggs were counted daily for 10 consecutive days. Females did not oviposit for longer than 6 days.

See ref. 5 for details of methods. *P*-values are given for the differences between the two experimental groups and the control (Tukey–Kramer test).

ing to ovaries are located (*A* in the figure). When a naturally terminated mating was followed by four 5-min copulations, however, no sperm were found within the basal part: the sperm cells were scattered in the fluid filling the



Axially sectioned spermathecae composed of basal part (**b**) and sac (black asterisk), showing distribution of semen (arrow). **A**, Control (three females processed); **B**, group 1 (four females). White asterisk, ovary. Magnification $\times 275$.

spermathecal sac (*B* in the figure).

It was impossible to estimate the number of sperm cells for comparison between the control and group 1, but it is unlikely that sperm had been removed in the course of the short copulations because the inseminatory canal is very narrow, about the diameter of a sperm cell⁴, and relatively long.

We doubt that there was a transfer of some substance that could selectively incapacitate the sperm of other males⁵ or cause its removal by the female⁶ during the first phase of copulation. Such substance, if deposited by the first four males in group 2, should have remained active and caused considerable decrease in female fecundity. But this was not the case (see table).

We conclude that during copulation in *C. berlessei* (and, presumably, in other acarid mites) males first transfer some substance which can push the previously deposited sperm away from the basal part of the spermatheca and the efferent

ducts leading to the ovaries, and only then sperm is transferred. The chance that sperm repositioned and dispersed in the spermatheca will reach the efferent ducts again must be very low. It seems possible that this sperm is decomposed and finally resorbed by microvilli rich walls of the spermatheca⁴.

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Terpenes and forest decline

SIR – Becker *et al.* imply¹ that reactions of terpenes with ozone could have a more important role in the formation of hydrogen peroxide than the disproportionation reaction of peroxy radicals, thereby contributing to forest decline. The importance of the oxidation of terpenes to the atmospheric H₂O₂ budget can be estimated by using the authors' reported molar yields and terpene fluxes from coniferous forests. Lamb *et al.*² reported α -pinene fluxes of 1.15×10^{-5} mol m⁻² h⁻¹ for a Loblolly pine forest (*T* = 30 °C). If one assumes a mixing height of 1 km and that β -pinene, Δ^3 -carene and *d*-limonene fluxes are similar, the resulting H₂O₂ production rate would be less than 6 p.p.t.v. (parts per 10¹² by volume) h⁻¹. The disproportionation of peroxy radical HO₂· + HO₂· → H₂O₂ would produce 25–700 p.p.t.v. H₂O₂ h⁻¹ (*k_r* = 5.5×10^{12} cm³ per molecule s⁻¹; *T* = 25 °C; relative humidity of 50%)³ at HO₂ levels of 10–15 p.p.t.v. (which are thought to be typical midday levels)⁴. Hence these calculations indicate that H₂O₂ production from the oxidation of terpenes may not be as important a source of atmospheric H₂O₂ as Becker *et al.* suggest.

The concentrations of reactants used by Becker *et al.* [*O*₃]₀ = 2–60 p.p.m.v., [alkene]₀ = 5–35 p.p.m.v.) are not relevant to the atmosphere. Concentrations of O₃ in rural areas are generally of the order of 30–70 p.p.b.v.⁵; concentrations of terpenes, both in absolute terms and relative to O₃ levels, in the ambient atmosphere are also much lower. Measurements indicate that total terpene

concentrations in forest air during the summer are of the order of 0.1–10 p.p.b.v.^{3,6}. Highest concentrations are generally found at night since convection and vertical transport are lower; total terpene concentrations at midday are seldom larger than 1 p.p.b.v. Fluxes of terpenes from forests are dependent on various factors, including the plant species, time of year and leaf temperature⁷.

Becker *et al.* also noted that H₂O₂ production increased with addition of water to the reaction chamber. They attributed this to the acid/base catalysed reaction of the Criegee radical with water to form H₂O₂. However, with their experimental design and the blank tests reported, one cannot exclude reactions of water and O₃ on glass surfaces to account for a portion of the increases in H₂O₂ concentrations, even though "rapid" appearance of H₂O₂ was observed. Such reactions are known to cause serious errors in H₂O₂ measurements^{8,9}.

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BECKER *ET AL.* REPLY — Ross *et al.* imply that the reactions of terpenes with ozone do not play an important role in the formation of hydroperoxides in the atmosphere and consequently in forest decline. Their estimation of the atmospheric hydrogen-peroxide budget using total terpene concentrations of about 1 p.p.b.v. is incorrect. The total terpene concentrations at midday are seldom higher than 1 p.p.b.v. because of the high oxidation rates. The estimation cannot be simply deduced from our re-

ported values from the H₂O₂-yield table; these values were measured under high concentrations of O₃ and terpenes, which result in high losses for the Criegee biradical by reactions with other species (such as HCHO and higher aldehydes). The estimation of the H₂O₂ formation rate, resulting from the reaction of the biradicals with water vapour, is only possible by a full kinetic simulation of forest-air chemistry. These simulation calculations must include the emission rates of the terpenes^{10,11}, the rate constants for the reactions R₂COO + H₂O → H₂O₂ and the yield for H₂O₂ formation for the different terpenes.

Ross *et al.* mention that the high concentrations used in our experiments were not relevant to the atmosphere. The relevance to atmospheric chemistry is given not by the concentrations used but by the occurrence of the reaction R₂COO + H₂O → H₂O₂ in atmospheric photo-oxidant systems. The reactions were carefully analysed by evaluating "concentration-independent" kinetic parameters in the case of C₂H₄ and tetramethylethene and O₃. The clearly established new reaction of water with biradicals seems to be an important H₂O₂ source in forest air and can possibly contribute to forest decline.

Heterogeneous formation of H₂O₂ on glass surfaces was ruled out by extensive blank tests. Details of these tests and their consequences for the kinetics of the reaction of the Criegee biradical with water were not reported in our paper¹. Blank tests with even higher ozone concentrations (up to 400 p.p.m.v.) in the presence of water vapour (1–10 torr) have shown no H₂O₂ formation. References 8 and 9 are irrelevant to our work. These describe experiments in which O₃ was bubbled through water. H₂O₂ formation in such systems can lead to interference in wet-chemical methods for H₂O₂ measurement, but certainly plays no role in our experiments. The high H₂O₂-yields recently observed with the wet-fluorescence method in the reactions of biogenic hydrocarbons with ozone in the presence of water vapour¹² are possibly due to such interference. An

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enhanced O_3 - H_2O surface chemistry in the presence of unsaturated hydrocarbons was not observed. No change in the stoichiometry (terpene/ozone) was observed on varying the water vapour, terpene and ozone concentrations.

In conclusion, it should now be clear that all the points mentioned by Ross *et al.* concerning the heterogeneous formation of H_2O_2 and the experimental conditions are irrelevant for our work. Our aim was to report the occurrence of a new type of reaction that results in the formation of H_2O_2 , also under conditions prevailing in forest air. Ross *et al.*'s estimation of the H_2O_2 budget in forest air due to the reaction of the Criegee biradicals with water vapour has not been correctly evaluated and may lead to wrong results.

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Beneficial ghee?

SIR — Aneja and Murthi's Scientific Correspondence¹ may encourage people, especially Asian Indians, to eat more ghee (anhydrous milk fats of cow and buffalo). But the authors show only that ghee contains 2–3% of conjugated linoleic acid (CLA), a chemical that may have anti-carcinogenic properties.

CLA in isolated (pure) form may indeed be anti-cancerous, but solid evidence is necessary to show that this property is retained when it is mixed with a large excess of saturated milk fats. The evidence for the anti-carcinogenic property of CLA is based upon inhibition of the initiation of mouse epidermal tumours (on shaved dorsal skin area) promoted by DMBA (7,12-dimethylbenz[a]anthracene)². It is not known whether CLA would exhibit this property in mice, let alone in humans, if administered orally and especially when combined with a large dose of saturated fat.

It also remains to be seen whether the beneficial effects of CLA (if proven) outweigh the known hazards of a 49-fold excess of saturated fat in ghee. Unless these concerns are adequately investigated, an unsagacious consumption of ghee might turn out to be a fat mistake.

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Spatial maps

SIR — Halligan and Marshall¹ describe a patient (T.M.) who, following a lesion in the right hemisphere of the brain, exhibited left neglect in near (peripersonal) but not far (extrapersonal) space. As this dissociation has also been demonstrated in animal studies², it seems to establish an important link between human models of attention and those derived from neurophysiology. But there are some important caveats.

The problem of relating physiological data to those obtained from human studies is that it is rarely possible to control the locus and extent of lesions in humans. Halligan and Marshall suggest that the dissociation exhibited by T.M. may have occurred because the (hypothesized) structure controlling attention in extrapersonal space (area 8) was spared. However, as a consequence of damage to another part of the same anatomical circuit, the posterior parietal lobe, we believe reciprocal connections between these areas were almost certainly disrupted. Thus, the nature of T.M.'s lesion precludes extrapolation from behavioural observation to neuroanatomy.

This leaves the problem of explaining the apparent dissociation of symptoms in T.M.. One could assume that dissociation of neglect in near and far space might occur in other patients tested under similar conditions. As the paradigm Halligan and Marshall used in extrapersonal space has not been applied to other patients (or normal controls), this issue remains unresolved. But given the previous failure to demonstrate a dissociation using another paradigm³, we believe the observations may be biased by methodological inconsistencies.

The kind of response required of T.M. in peripersonal space was different from that required in extrapersonal space. In the latter, "T.M. was asked to make a single movement to the centre of the line" (page 499 of ref. 1) using a light pen, before indicating his judgement. This is clearly a different response to that required in peripersonal space, where T.M. was apparently free to approach the subjective midpoint from any position and make adjustments until satisfied. It is important to consider the extent to which postural abnormalities induced by severe left hemiparesis may have biased control of the ipsilesional arm in the dart-throwing condition.

Finally, directional hypokinesia (impaired ability to direct responses toward the contralesional side of space) may have biased bisection errors in peripersonal but not extrapersonal space. Arm movements in peripersonal space, which involve proximal musculature, are impaired in patients with neglect⁴. By contrast, pointing a light

pen at a distant (extrapersonal) target requires only distal manipulation. Any manifestations of directional hypokinesia would be markedly attenuated under such circumstances.

As the directional impairment in neglect can be modified by different response factors, future attempts to elicit dissociation must use identical paradigms in peripersonal and extrapersonal space. If a motor response is required to elicit the dissociation (as Halligan and Marshall suggest), the problem of finding an appropriate paradigm will be difficult, if not impossible. In any case, the kind of data derived from human lesion studies may not be sufficiently sensitive to draw the distinction.

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MARSHALL AND HALLIGAN REPLY— We agree with the neuroanatomical caveats raised by Mattingley and Bradshaw: T.M.'s lesion was large and the link we drew with studies in monkeys², although supporting our hypothesis, was tentative. Nonetheless, the fact is that T.M. showed neglect in near but not far space¹, although the question of why this was so remains open.

The speculations that Mattingley and Bradshaw put forward are not convincing. For all trials in all conditions, T.M. sat upright in his wheelchair. Each trial commenced with his right arm placed on the right armrest of the chair. Each response, with pen, lightpen or dart, accordingly began from this position. When a pen was used, T.M. was encouraged to make a single, overt movement to the centre of the line (as he was in all other response conditions). In future studies, it would be desirable to make a video record of patients' performance⁵; but if T.M. had made more "adjustments until satisfied" in peripersonal space, surely his accuracy would have improved (and given the opposite result to that found)⁶? We observed no "postural abnormalities" when T.M. was throwing darts (other than that he was chairbound with a left hemiparesis!) and cannot readily imagine how such abnormalities would improve his performance.

With respect to the vexed question of directional hypokinesia, the comment of Mattingley and Bradshaw is seriously misleading. In the paper they cite⁴, Heilman *et al.* found that, compared with control subjects, patients with left neglect showed a small but significant increase in reaction time to initiate a leftwards movement of the arm. There was no suggestion that the patients could not perform the task, nor that the involvement of proximal musculature *per*

se was implicated in the increased initiation latencies. In our experiment, all conditions required the use of both proximal and distal musculature; when pointing the light pen at the distal targets, T.M. always first raised his arm from the armrest, just as he did in all other conditions. If the subsequent direction of pointing was controlled primarily by hand and finger movement, why would T.M. not adopt the same strategy (where possible) in near space? The significant difference reported between conditions 2 (far space) and 5 (near space), both of which use a light pen, is not predicted by the directional hypokinesia hypothesis. Furthermore, as we originally stated, T.M. had no difficulty touching left-sided parts of his body.

We share our critics' bemusement at our results – we were ourselves astonished to find such a strong effect. But we recommend that Mattingley and Bradshaw attempt to replicate them. We have subsequently tested two further patients with left neglect in this para-

digim – one (with a temporal lesion) showed the effect seen in T.M., the other (with an occipito-temporal lesion) did not. We do not share the scepticism of Mattingley and Bradshaw about constructing better controlled paradigms. Their point about distal versus proximal musculature, for example, could be overcome by strapping the patient's wrist and attaching a laser pointer to the back of the hand.

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C₆₀ zeolites?

SIR — Single crystals of C₆₀ have been shown recently¹ to have close-packed structures, and there has been speculation² about the effects of pressure on this structure. I would like to draw attention to a hypothetical, high-pressure cubic crystal structure (see figure) which retains the C₆₀ groups, but in which all the carbon atoms are in 4-coordination, achieved by bonding of each molecule to 14 neighbours. This framework structure is much denser than the molecular crystal and also represents a new possibility for a zeolite-type framework.

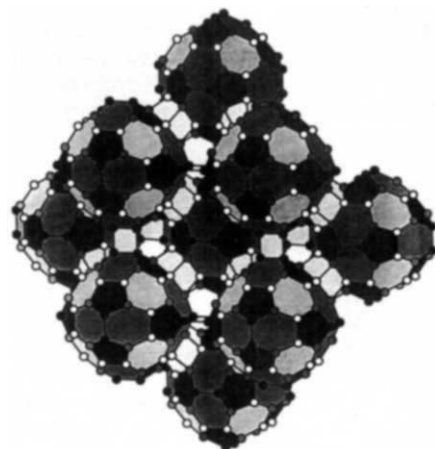
The symmetry group of molecular C₆₀ is the icosahedral group *I_h*. If the molecules are packed in a crystal in an ordered way, the highest possible symmetry of the cluster is *T_h*=*m3* (the maximal crystallographic subgroup of *I_h*). As the order of *T_h* is 24, there must be at least three distinct C-atom positions in the crystal.

In the structure shown in the figure, the C₆₀ groups are arranged with their centres forming a body-centred cubic lattice, 8 of the 20 hexagonal faces being normal to the 3-fold axes of the lattice. If the polyhedra at the body centres of the cubic cell are rotated by 90° about a 2-fold axis with respect to those at the

corners, the resulting space-group symmetry is *Pm3n* and the point symmetry at the centre of the clusters is *T_h*. Pairs of hexagonal faces on adjacent polyhedra now form slightly twisted hexagonal prisms. If the length of the cell edge is $a = 6.3478d$, where d is the C–C bond length on each C₆₀ molecule, the shortest C–C distance between an atom on one hexagonal face and that on the opposite one is equal to d , so that 48 of the 60 atoms have four nearest neighbours. The remaining 12 atoms are a distance $1.49d$ from their mirror images on polyhedra in adjacent unit cells.

Only small shifts of atom positions (without change of symmetry) are needed to give all 60 atoms four equidistant neighbours. There are nine structural parameters (a and the atomic coordinates listed in the table), but only seven independent nearest-neighbour distances so that, after these are fixed, the structure still has two degrees of freedom. The parameters in the table were determined by fixing the bond distances at $d = 1.54 \text{ \AA}$ (appropriate for C–C single bonds) and minimizing 'non-bonded' repulsion energies. The latter were taken to be proportional to $\exp(-\alpha r)$ where r is the separation and $\alpha \approx 3.0 \text{ \AA}^{-1}$ for C...C interactions³.

This shift produces a lattice parameter of $6.1916d = 9.54 \text{ \AA}$, and the shortest



A fragment of the structure described in the text. Filled circles are C(1), shaded circles are C(2) and open circles are C(3). The lighter-shaded hexagons are parts of hexagonal prisms.

non-bonded distance is $1.32d$; the positional parameters are given in the table. If C₆₀ could be induced to crystallize (possibly at low temperature) with a body-centred cubic structure of weakly interacting 3-coordinated molecules with an effective radius of $5.02 \text{ \AA}$, one might then expect that increasing pressure would transform the structure to the 4-coordinated one with a 45% decrease in volume. The decrease in volume per unit cell is approximately 40% relative to the close-packed molecular structure. No bonds need to be broken to effect this transition.

The structure is also of interest as a means of space filling by polyhedra. In addition to the two truncated icosahedra, the unit cell contains eight hexagonal prisms (symmetry *D_{3h}*), six distorted truncated octahedra (symmetry *D_{2d}*) and six dodecahedra (symmetry *D_{2d}*) with four quadrilaterals, four pentagons and four hexagons as faces. Considered as a 4-connected net, the symbols⁴ of the vertices are C(1) : $4.5.6^4$, C(2) and C(3) : $4^2.5.6^3$.

Since writing this letter, the structure of a body-centred cubic intercalate, Cs₆C₆₀, has been described⁵. This has lower symmetry *Im3* and all truncated icosahedra are in the same orientation. The optimum 4-coordinated structure with this symmetry, analogous to that shown in the figure, has very similar coordinates: $a = 6.2052d$, C(1): 0.0, 0.4194, 0.0806; C(2): 0.1275, 0.3444, 0.1445; C(3): 0.0806, 0.2557, 0.2706.

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ATOMIC COORDINATES FOR THE NEW C₆₀ CRYSTALS

Atom	x	y	z	R/d
C(1)	0 (0)	0.4192 (0.3823)	0.0808 (0.0788)	2.64 (2.48)
C(2)	0.1299 (0.1274)	0.3424 (0.3337)	0.1384 (0.1575)	2.42 (2.48)
C(3)	0.0808 (0.0788)	0.2619 (0.2549)	0.2695 (0.2850)	2.38 (2.48)

C(1) is in positions $24k$, C(2) and C(3) in $48j$. Coordinates in parentheses are for regular truncated icosahedra. R is distance from the centres of the polyhedra (at (0,0,0) and $(\frac{1}{2}, \frac{1}{2}, \frac{1}{2})$) and d is the C–C bond length.

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The power of invention

A. Rupert Hall

The Magic of Numbers and Motion: The Scientific Career of René Descartes.
By William R. Shea. Watson Publishing International: 1991. Pp.371. \$54.95.

No one would deny the immensity of René Descartes' achievement in philosophy and mathematics, or the huge influence that he had on his successors. But in natural science the situation is different, although no one alive between 1650, the year of Descartes' death, and 1680 would have seen the distinction. Even into the second quarter of the eighteenth century, Descartes' views on the fabric of the Universe were still influential, having become modified by such neocartesians as Huygens and Leibniz; yet Huygens had called Descartes' world 'science fiction' and Leibniz had proved that the man had not correctly understood the very foundation of his physics, the concept of motion. Worse, newtonian mathematical physics and British empiricism had utterly subverted the world that Descartes had so cunningly contrived by *a priori* reasoning. "I have described the Earth and the entire visible Universe", Descartes declared, "on the model of a machine, without considering anything beyond figures and motion." But neither cartesian concepts nor cartesian mechanics stood up long to scrutiny, and the cogs of this machine simply would not turn. (Contrary to common belief, cartesian coordinates were not Descartes' invention.)

Shea traces the intellectual path taken by Descartes from his first discussions with Isaac Beeckman at Breda in 1618. Descartes was then a gentleman-volunteer in the army of Prince Maurice, but it is impossible to imagine that he was ever serious about soldiering, and he was soon deep in the analysis of mathematical and scientific problems that, together with his work on epistemology and metaphysics, would occupy the rest of his life. The fruit of his labours was a concept of physical action, which in principle but not practice was mathematical and could be reduced to the idea of the pressure (motion) of particle on particle. Shea does not ignore the weakness of Descartes' working principles, which could almost be a play on words: if there is nothing (nothingness) between bodies, they must be in contact; potential motion (pressure) is the same as real motion, and so on. Nor does Shea conceal that after 25 years of intellectual

odyssey, Descartes had become grudging in gratitude to others (for example, to Beeckman) and was ready to appropriate their ideas as his own. Yet his power of invention was so immense that he had little need for the notions or even information of others. Like others, Shea is



Portrait of Descartes by Frans van Schooten (1644). Descartes praised the portrait, "although the beard and the clothes bear no resemblance to reality".

unable to explain why Descartes could not accept William Harvey's account of the heart, which was stripped of non-mechanistic features. Instead, Descartes preferred an explanation of his own, which could not only be refuted by simple observation but was also mechanically absurd. In fact, Descartes had not understood Harvey's discovery of a great flow of blood about the body, and his claim that it was only those "ignorant of the force of mathematical demonstrations" who would reject his version was just vacuous bluff.

Such sweeping intellectual claims perhaps more readily bluff the young than those settled with comfortable ideas. For the young of the mid-seventeenth century, that time of bursting bonds, Descartes promised innovation, enlightenment and certainty, in much the same way as Darwin, Marx and Freud seemed to do several generations later. Descartes offered a key to

the great mystery of the cosmos, entertaining the idea of a God-like power of creation (for he alone could imagine causes of things that could not be anything but the truth). In *Principia Philosophiae* (1644) he set out his own key to nature — "mechanical philosophy" — an approach that promised an end to verbal quibbles, meaningless distinctions, dormitive faculties, and even the tedious technicalities of astronomers and anatomists. By thinking, a man exists; by thinking clearly and distinctly, he can know all he needs to know. It is not surprising that this message of logical idealism for a time carried all before it.

What a magnificent prospect, the limits of knowledge within sight. If Harvey differed from Descartes, so much the worse for his experiments, which so easily mislead.

Of course there is much more to Descartes than apriorism. There is his strong sense of religious purpose, about which Shea writes; his touch of mystic platonism; even a smattering of baconian curiosity. The best of his science was with light and optics. We can forgive Descartes his prevarications about the physical cause of the sine law in refraction for the sake of his superb investigation of the rainbow. Newton was notoriously unkind to Descartes here, whereas Shea is perhaps a little ungenerous to Descartes' predecessors, for cracking the mystery of the rainbow was one of the huge technical successes of mediaeval science. Descartes' long concern with light and optics is a main theme in the book and it is handled excellently.

Most treatments of cartesian science have been dull, misdirected or reverential; here we have an account that is lively, critical and thoroughly informed by both ancillary sources and Descartes' correspondence. The greater technical depths can be plumbed elsewhere, and it is a pity that the geometry has gone a little astray on pages 50 and 53 (it is readily amended). Nonetheless, the book is admirable for being historically contextual, sympathetic yet detached about Descartes' intellectual programme, and clearly organized (only here and there does the chronological thread become a trifle hard to follow).

I wish I could have read this book long ago: a comprehensive and fascinating introduction to a man whose writings must be absorbed before later seventeenth-century science, not least Newton's, can be fully appreciated. □

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Humans in the balance

Robert Attenborough

The Rise and Fall of the Third Chimpanzee. By Jared Diamond. *Hutchinson Radius*: 1991. Pp. 360. £16.99.

If Professor Diamond's Gibbonesque title hints at stunning new discoveries in cryptozoology or palaeontology, then the hint has misled. His third chimpanzee is Aristotle's political animal, Oakley's toolmaker, Morris's naked ape, Tobias's tottering biped, Wilson's promising primate, Passingham's human primate. . . in short, you and me. This is a book about the evolutionary origins of our species, its development and special features (attractive and otherwise), and its future.

As a crisp encapsulation of the human condition, 'third chimpanzee' is a more apt phrase than many; nonetheless, it is a little strained. Our kinship with chimpanzees is indeed now shown by molecular evolutionists to be even closer than anatomists and palaeoanthropologists took it to be: but by any measure we remain much more strongly differentiated from the common and pygmy chimpanzees than they are from each other; and if (as it seems) we and chimpanzees are phylogenetically closer to each other than to gorillas, it is probably not by very much. Differences between chimpanzees and humans in DNA and protein are small (1.6 per cent in the DNA study that Diamond quotes), which shows not just that we are phylogenetically close, but also that relatively few genes can produce large phenotypic differences. Diamond concedes much of this, although he also maintains, unconvincingly to my mind, that a properly revised taxonomy (*Homo troglodytes* for chimpanzees, and so on) would have large conceptual and ethical consequences.

The book is, however, too diverse in content to be judged simply on the idea crystallized in its title. It deals with aspects of humans as various as their evolution, life cycle, unique characteristics, dispersals and conquests, and the threat to their survival posed by destruction of biota and habitat. Most of the chapters are versions of articles originally written for *Discover* or *Natural History*; and as the book proceeds, their separate origins become more apparent despite linking sections.

Whether viewed as a whole or as a collection of essays, his book confirms Diamond as an impressive scholar and popularizer. He makes effective use of his own areas of expertise, with birds

providing many of his behavioural and ecological examples — a refreshing change from primates. More striking is how acutely he observes other fields. I can find no serious fault with his facts about primatology, human evolution and archaeology; his sources are good and often very recent; and with minor exceptions his overviews are up to date and his interpretations defensible. One might ask whether the "great leap forward" (Mao's phrase borrowed to describe the cluster of human behavioural innovations suddenly emerging around 40,000 years ago) will turn out to be an artefact of working near the limits of radiocarbon dating: nevertheless, even here Diamond's interpretation is broadly consistent with the evidence that is currently available.

On some controversial issues Diamond justifiably takes sides. He favours rapid replacement from Africa rather than multi-regional continuity for the evolution of anatomically modern humans; he adopts late dates for the peopling of the Americas; and he suggests a Bronze Age steppe rather than a neolithic Near Eastern origin for Indo-European languages, with the horse as their vehicle for dispersal. Generally, he outlines the opposing view as well as his own, and gives reasons for his opinions, although not in detail. On the peopling of the Americas, for instance, there is too little detail for the reader to form an independent view; yet Diamond's adoption of the dominant orthodoxy here is crucial to his interpretation of faunal extinctions.

On other fields Diamond is sometimes less sure-footed. I am unpersuaded by his explanations for racial differences (Darwin's sexual selection hypothesis), assortative mating for physical traits (direct mate choice) and drug abuse (Zahavi's sociobiological handicap principle). And the chapter on art is disappointing, being anthropologically unsophisticated. More generally, a worrying progressivism and cultural absolutism (if that is the opposite of relativism) creeps into many of the interpretations of cultural behaviour, past and present, despite his explicit caution against this way of thinking in the chapter on agriculture. There is also a general tendency to interpret cultural behaviour glibly in natural-selection terms.

Two small slips warrant a mention: a sociobiological relationship is misstated on page 76; and a myth about the first outside contacts with highland New Guinea being in 1930 is repeated on page 205 (there were visits by German missionaries from 1919).

Diamond's approach is semipopular, neither as racy as Morris's, nor as scholarly as Passingham's. His prose is accessible, colloquial and literate, though

not without clichés. The stylistic devices that Diamond uses to ingratiate himself with the readers and to hold their attention can obtrude at times, but if they bring him a wider audience then all the better. The costs of this approach include some selectivity and simplification, and the omission of references from the text. It is an irony of the genre that where thorough sourcing is most needed, readability considerations permit no more than reading suggestions relegated to endnotes.

Nonetheless, this is an enjoyable, stimulating and audacious book, a showcase for Diamond's admirable ability to keep abreast of so many fields of research. And in disseminating his knowledge, he achieves his wider aim of consciousness-raising about the extinction we are causing other species, and the impact that this destruction might have on our own prospects of survival. □

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New in paperback

There is something for everyone's holiday relaxation in the crop of books newly published in paperback ■ *The Search for Eve* by Michael H. Brown is a journalist's account of the hunt for the mother of us all. J. S. Jones described it as "a racy tale about the studies of the human race and about the quarrels of those who study it" (*Nature* **345**, 395; 1990). Published by HarperCollins, price is \$10.95. ■ Those contemplating taking on Las Vegas in their summer break should read Thomas A. Bass's *The Newtonian Casino*, an insider's revelation of how a group of physicists and computer buffs sets out to beat the house at roulette with computers small enough to fit into their shoes (for review see *Nature* **345**, 776; 1990). Published by Penguin, price £5.99. ■ A less ambitious challenge is *The Lady and the Tiger?* by Raymond Smullyan, a collection of problems and paradoxes which lead to the very heart of Gödel's theorem (Oxford University Press, £6.99). ■ Also from Oxford comes *The Threat and the Glory* by Peter Medawar and *A Very Decided Preference* by his wife, Jean Medawar. The first contains Peter Medawar's Reith lectures and several of his essays, while the second was described by Alex Comfort as being "an unsentimental tribute" to life with the great man (*Nature* **346**, 709; 1990). Both are priced at £6.99. ■ In *The New Age: Notes of a Fringe-Watcher*, Martin Gardner continues his noble crusade against pseudoscience and the paranormal with pieces taken from *Skeptical Inquirer*, *New York Times*, *Discover* magazine and other publications (Prometheus, \$15.95). ■ Finally, everyone would be advised to absorb at least a few of the thoughts in Alan L. Mackay's impressive *A Dictionary of Scientific Quotations* (Adam Hilger, £9.50), for in the words of Albert Einstein "The whole of science is nothing more than a refinement of everyday thinking." □

In the beginning

Michael Rowan-Robinson

The Shadows of Creation: Dark Matter and the Structure of the Universe. By Michael Riordan and David N. Schramm. W. H. Freeman: 1991. Pp. 278. £18.95.

IN his 1977 classic, *The First Three Minutes*, Steven Weinberg took as his starting point the moment one hundredth of a second after the Big Bang when the first stages of cosmological nucleosynthesis began. The basic features of the Big Bang model have been well understood from that point onwards for more than 20 years. The microwave background radiation, discovered by Arno Penzias and Bob Wilson in 1965, demonstrates that for the first 100,000 years of its history the Universe was dominated by radiation, the 'fireball' phase. Only when this radiation had cooled sufficiently could ordinary matter begin to respond to the gravity field generated by the ripples in the density distribution that our existence proves must have been present. This microwave radiation, responsible for at least part of the noise on our television screens when broadcasting ceases, gives us a direct image of the Universe as it was only 100,000 years after the Big Bang. But the radiation is incredibly smooth: so just how could the lumpy Universe that we see today, of which our bodies with a density 10^{30} times the average are the epitome, have arisen from such a beginning? That has been one of the key questions of the past decade.

Although the microwave background radiation represents our earliest direct measurement of the Universe, the nuclear physics of 20 years ago allowed us to extrapolate back to a time one hundredth of a second after the Big Bang. The prediction of the abundances of the light-elements, helium, deuterium and lithium, was one of the triumphs of Big Bang cosmology in the 1960s and 70s. The remarkable development of the past decade, which is brilliantly expounded in this new book, has been to push our understanding of the history of the Universe back in time a further 10^{33} times to 10^{-35} seconds after the Big Bang. Some of the ideas involved are so bizarre and outlandish as to baffle all but the most dedicated particle physicist. Yet, strikingly, they have been widely accepted by theoretical cosmologists, despite an almost complete absence of experimental confirmation.

The Shadows of Creation is a superb book, perhaps the best on a cosmological subject since *The First Three Minutes* (Basic Books), certainly, more illuminat-

ing and relevant than the fêted *Brief History of Time* (Bantam, 1988) by Stephen Hawking, who, incidentally, writes the preface to the new book. In a nutshell, its authors describe how theories for the unification of the forces of nature led first to the idea of an inflationary Universe and thence to a model of a Universe filled with some new kind of dark matter so far undiscovered. The first step along this road, the unification of the electromagnetic and weak-nuclear forces by Steven Weinberg and Abdus Salam, was powerfully vindicated with the discovery at CERN in 1983 of the W and Z gauge bosons which 'mediate' this unified electroweak force.



"THE BIG BANG? BELIEVE ME, IT WAS VERY, VERY, VERY, VERY, VERY, VERY BIG."

In modern particle physics, all forces can be thought of as being due to the exchange of a mediating particle, from the photon for the electromagnetic force to the gravitino for gravitation. Experimental confirmation of the idea that the strong nuclear force can be unified with the electroweak force has yet to be achieved, but particle physicists and theoretical cosmologists take it as read that these forces were unified before the first 10^{-35} seconds after the Big Bang. At that instant, a phase transition is believed to have occurred, which left our bit of the Universe with an immense vacuum energy density that then behaved like a huge repulsive force, suddenly inflating the Universe exponentially by a factor of 10^{50} or so. 'Inflation' solves several metaphysical cosmological problems and would be expected to leave the Universe with a density 10–100 times that of ordinary matter today. It is this 'dark matter' that is the shadow of creation.

The dark matter can also solve the problem of how gravity can make galaxies and ourselves, starting from the smooth state revealed by the microwave

background radiation 100,000 years after the Big Bang. Riordan and Schramm weave us through the fascinating story of how particle physicists, cosmologists and astronomers have orbited about one another to establish a theory on the sands of the invisible and (so far) undetectable. The exposition of the nuclear physics is brilliant and will give the non-expert reader a real sense of getting to grips with the remarkable developments of the past decade. So many different theories about the nature of dark matter are presented, however, that the reader may find it hard to get a clear picture of what is believed. If 'superconducting cosmic strings' have been eliminated by recent observations, do we need to know about them in the first place? Perhaps it is a virtue, though, that the authors do not go overboard for any particular theory about the nature of dark matter.

There have been some considerable developments in cosmology since the book went to press. The galaxy redshift surveys carried out by my colleagues and myself with the Infrared Astronomical Satellite show that, on the one hand, the motion of our Galaxy through the microwave background radiation can be understood provided that the density of the Universe is indeed as high as predicted by inflationary cosmology. On the other hand, there is more structure on large scales than is predicted by one of the most popular dark-matter theories of galaxy formation, that of 'cold' dark matter. I was delighted, incidentally, by the figure on page 149 of the book showing a monster 'Great Attractor' behind the Hydra-Centaurus cluster, because when I recently announced that surveys with the Infrared Astronomical Satellite did not show this, a prominent Great Attractor supporter responded bitterly, "That [the existence of such an object] was not what I meant at all. That was not it at all."

I was surprised by the authors' implication that gravitational lenses have not yet been detected, for a few good examples have been known for several years. And dark halos of spiral galaxies stabilize the disc against the formation of a bar, not against disruption.

Well-written and excellently illustrated, this fascinating and inspiring book will give the general reader valuable insight into the way particle physicists and cosmologists are thinking today. □

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Chinks of light

Rodney Loudon

Atoms and Light: Interactions. By John N. Dodd. Physics of Atoms and Molecules Series. Edited by P. G. Burke and H. Kleinpoppen. Plenum: 1991. Pp.247. \$78.

THE relationship between the classical and quantum theories of light intrigues almost every physicist trying to reconcile its classical-wave and quantum-particle behaviours. Strong similarities exist between the two theories: Maxwell's equations are valid in both the quantum and classical schemes, and the boundary conditions controlling the behaviours of light beams are common to both. Optical systems are therefore described by identical sets of spatial modes in the two theories, which thus predict the same distributions of intensity in optical experiments. Differences arise only when experiments are performed in which the operator characters of the field vectors in the quantum theory are brought into play by the measurement process. The noncommuting properties of the field operators can then produce such typical quantum-mechanical effects as Heisenberg's uncertainty limitations on the accuracies of measurements and unavoidable perturbations of the measured system.

It turns out that these quantum effects are difficult to observe in practice, and the results of most optical experiments can be perfectly well accounted for by the classical theory of light. Even experimental results that were once thought to demand a quantum interpretation have yielded to theories in which the optical field is treated classically but the full quantum theory is applied to any atoms whose transitions are used in making the measurements, the so-called semiclassical theory. Such is the case with the photoelectric effect, which was regarded as the exemplar of the quantum interpretation, but can in fact be accounted for semiclassically.

Nevertheless some optical effects do require the quantum theory for a quantitative explanation. So although the results of all experiments performed to date are in accordance with the quantum theory of light, only a subset (albeit a large one) accords with the classical interpretation. The nonclassical effects often require feeble light for their observation, where the light has a chance to display its quantum granularity, but such light does not necessarily show quantum effects. For example, careful experiments show that the fringe patterns in a Young or Michelson interferometer do not detectably change

however feeble the intensity of light, provided that there is a compensating increase in the recording time. On the other hand, a light beam divided by a simple beam splitter shows extreme particle-like effects, with each incident quantum being directed into either one output arm or the other, but never being separated into two half quanta. Indeed, it is the correlation effects between two or more light beams that provide a rich variety of quantum phenomena.

Dodd is a proponent of the classical theory of light and the semiclassical methods of treating its interaction with atoms. He presents these theories straightforwardly, and I criticize only his insistence on using the ordinary frequency ν instead of the angular frequency ω , trivial perhaps, but the resulting high powers of 2π complicate many of the equations. The main part of the book explores the extents to which the semiclassical theory can describe results that have been conventionally regarded as needing a quantum interpretation. The

approach is honest, with shortcomings in the semiclassical theory clearly identified. Modifications that are needed to coax the theory towards the desired results and that are sometimes seemingly arbitrary and difficult to justify, are properly discussed. At the end of the book, Dodd describes his novel views about the nature of the classical and the quantum theories.

The book will appeal mainly to those whose interests are in stretching the applicability of semiclassical theory and in finding chinks in the armoury of quantum optical effects that yield them amenable, after all, to semiclassical theory. Most will probably conclude that if they really want to understand the whole range of optical experiments and predict new phenomena to be observed, then they had better come to terms with the quantum theory of light. □

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Body building

Susan E. Evans

Functional Chordate Anatomy. By Ronald G. Wolff. D. C. Heath and Co., 125 Spring Street, Lexington, Massachusetts 02173, USA: 1991. Pp. 752. \$50.

How do you breathe if you have no diaphragm and your ribs are fused into a rigid shell? Turtles solve the problem by shunting their internal organs around and by changing the volume of soft pockets at the bases of their limbs. Why do cats get stuck in trees? Because their claws are designed to grip on the way up and a cat must learn to come down in reverse.

Functional Chordate Anatomy covers similar ground to its predecessors, beginning with a history of anatomy, and introducing systematics and embryology before launching into detailed accounts of the main body systems. It differs, however, in the breadth of its coverage. The book contains all the classical stories of comparative anatomy — aortic arches, heart and kidney structure, limb patterns and the like — but Wolff has researched his subject well and has included a wealth of additional information previously available only in the primary literature. Each of the main chapters begins with an account of development and ends with a phylogenetic overview. Structure is related to function in the context of individual lifestyles. This is refreshing; some texts leave the impression that non-mammalian amniotes are simply imperfect prototypes of

the mammalian end model.

The book is well-organized and clearly written, with good chapter bibliographies and an extensive glossary. It is, to coin a vogue term, 'user-friendly'. A book as comprehensive as this, however, will inevitably contain small errors (wandering labels for example) and omissions. If I have one serious criticism, it is that the text is not always internally consistent. The digits of the bird hand, for example, are numbered 1, 2 and 3 in one chapter, but 2, 3 and 4 in another. There are genuine differences of opinion between palaeontologists, comparative anatomists and developmental biologists on the homologies of these digits, but a note of explanation might have aided the bemused reader. The inconsistency also extends to the systematic section. Wolff presents a long, overly detailed and, in many ways, outdated list of chordate taxa. Here the text is better, being clearly derived from a different source, but in some places it directly contradicts the preceding list, the description of the relationships of frogs and salamanders being an example.

Nonetheless, Wolff is to be congratulated for having produced a very readable book, of use not only to all with an interest in zoology and anatomy, but also to those seeking a wider perspective, such as medical students and developmental biologists. He has shown that comparative functional anatomy is an active and exciting field with plenty of questions left to be answered. □

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A 'unified theory' of prion propagation

C. Weissmann

There is now very persuasive evidence that the transmissible agent for spongiform encephalopathies such as scrapie, consists of a modified form of the normal host protein PrP^C, devoid of any nucleic acid. On the other hand, because there are many different strains of scrapie agent with distinct phenotypes which can be propagated in animals homozygous for the PrP^C gene, it has been suggested that a nucleic acid must be a component of the agent. Can the two views be reconciled?

Two men asked a rabbi to settle their dispute. After listening to the first man's presentation the rabbi announced, "I find that you are right". The second man, dismayed, protested, "But rabbi, you haven't heard my side yet", and went on to present his case. The rabbi said, "Now that I have heard you, I find that you are also right". The rabbi's assistant interjected, "But rabbi, they can't both be right", to which the rabbi responded, "You are right, too."

THE nature of the transmissible agent that causes spongiform encephalopathies, such as scrapie or bovine spongiform encephalopathy in animals, or kuru, Creutzfeldt-Jakob disease (CJD) and Gerstmann-Sträussler-Scheinker disease (GSS) in man, is still controversial (for reviews see refs 1-7). The overall properties of the agent, which has been designated as prion⁸, differ from those of any known virus or viroid⁸⁻¹⁴ and early on gave rise to speculations that it might be devoid both of nucleic acid and of protein¹⁵, consist of protein only¹⁶⁻¹⁸, be a polysaccharide¹⁹ or a membrane fragment²⁰.

Several lines of evidence suggest that the prion is devoid of nucleic acid and identical with PrP^{Sc}, a modified form of PrP^C (refs 1-3, 21). PrP^C is a normal host protein²²⁻²⁴ encoded in a single exon of a single-copy gene²⁵ and is found predominantly on the surface of neurons, attached by a glycoinositol phospholipid anchor^{1,2,21,26}. PrP^{Sc}, in contrast to PrP^C, is relatively resistant to protease²² and accumulates intracellularly in cytoplasmic vesicles^{27,28}.

It has been proposed that PrP^{Sc}, when introduced into a normal cell, causes the conversion of PrP^C or its precursor into PrP^{Sc} (refs 21, 22, 29-31) by an unknown process which could involve chemical or conformational modification, during or after its synthesis. But the existence of many different strains of scrapie which can be propagated in the same inbred mouse line, and the apparent mutability of the agent³² are better explained by the updated nucleoprotein or virino hypothesis, which holds that the infectious agent consists of a nucleic acid genome and the host-derived PrP, which is recruited as some sort of coat^{22,33}. No evidence for such a nucleic acid has yet been adduced^{6,21,29,34}.

In this review I present a model (Fig. 1) which combines the essential features of both the 'protein only' and the 'nucleo-protein' hypotheses, accounts for most if not all experimental observations, does not invoke unknown mechanisms and makes testable predictions. It may also be wrong. In short, I propose that the infectious agent, as found in crude tissue extracts of diseased animals, consists of two components which together form the 'holoprion'. One component is PrP^{Sc} (the 'apoprion') which, even when devoid of nucleic acid, can cause transmissible disease, as proposed by Prusiner and his colleagues^{21,30}; the other is a nucleic acid (the 'coprion'), of which many variants can exist and which, by modulating the biochemical properties of PrP^{Sc}, determines its strain-specific characteristics. I propose that a nucleic acid of this type is normally associated with PrP^{Sc}, but is also present in uninfected host cells. When PrP^{Sc} devoid of coprion enters the cell, it may (but need not necessarily) recruit a cellular nucleic acid which can serve as coprion.

Coprion nucleic acid is replicated by normal cell enzymes, and replication is stimulated or even dependent on the presence of PrP^{Sc}. The aprion would thus be a pathogenic element whose primary structure is encoded by the host genome, and the coprion a facultative, transmissible genetic element.

Six propositions for transmission

The propositions presented in this section are supported by various lines of evidence, but not proven beyond reasonable doubt.

■ Nucleic acid is not essential for infectivity of scrapie prion preparations. This claim is based on the unusually small target size for ultraviolet and ionizing radiation^{17,35-41} (for other views see refs 42-44); the low ratio of nucleic acids to infectious units in highly purified prion preparations³⁴, and the failure to identify scrapie-specific nucleic acid in prion preparations or scrapie-infected brain tissue^{6,29,34,45-48}; and resistance of infectivity to treatment with agents modifying or damaging nucleic acids^{8,39,49}. Together, these data suggest that a nucleic acid ≥ 50 -100 nucleotides is not essential for infectivity (but see ref. 50 for a different conclusion).

■ Scrapie infectivity is associated with PrP^{Sc}. Purification of scrapie infectivity results in a preparation highly enriched with PrP^{Sc} (refs 51-54). Conversely, purification of PrP^{Sc} by affinity chromatography on an anti-PrP antibody column⁵⁵ leads to enrichment of infectivity. Gajdusek and his colleagues^{56,57} have reported that exclusion chromatography or polyacrylamide gel electrophoresis (PAGE) of PrP^{Sc} in the presence of SDS leads to the recovery of infectious agent with a relative molecular mass corresponding to that of PrP^{Sc}, which argues against association with a nucleic acid. These experiments provide support for the 'protein only' model, but there are contrary views⁵⁸⁻⁶⁶.

PrP^{Sc} is defined as a protease-resistant form of PrP^C with increased tendency to form aggregates^{22,53,54}. No chemical differences have so far been detected between PrP^{Sc} and PrP^C (refs 67, 21; N. Stahl, M. A. Baldwin and S. B. Prusiner, personal communication), but the ratio of PrP^{Sc} molecules to infectious units is about 10⁵ (refs 53, 54) and therefore the infectious entity, even if it is derived from PrP^C, may not have the same structure as PrP^{Sc} (ref. 3) and could be chemically modified.

■ The susceptibility of a host to scrapie infection is codetermined by the prion inoculum and the PrP gene. The role of the host genotype with regard to susceptibility to scrapie infection and to the course of the disease is revealed by two sets of findings. First, the incubation time for the same prion isolate may be different in distinct mouse strains, and is determined predominantly by the *Sinc* gene^{32,68-70}. *Sinc* is undoubtedly identical with *Prn-i* (refs 71-72), which is very closely linked to or coincident with *Prn-p*, the gene encoding PrP (refs 71-75). Short and long incubation times are associated with two distinct PrP alleles, *Prn-p^a* and *Prn-p^b*, respectively⁷⁶. It is noteworthy that the PrP coding region, consisting of 254 or 253 codons in rodents and man, respectively, is located in a single exon^{25,77} and that there is an uninterrupted reading frame on the antisense strand which completely overlaps it in all species examined⁷⁸.

Therefore, it is possible that *Prn-i* corresponds to the antisense coding region.

Second, when prions are transmitted from one animal species to another, disease often develops only after a very long incubation period if at all; however, on serial passaging in the new species, the incubation time may decrease dramatically and then stabilize. This so-called species barrier⁷⁹ can be overcome by introducing into the recipient host the PrP transgene from the prion donor^{30,80}. Thus, some strains of mice containing hamster PrP transgenes, when inoculated with hamster prions, show incubation times as short as 75 days, rather than 500 days or more, as in the case of nontransgenic controls⁸⁰. Moreover, prion preparations from mice carrying hamster PrP-transgenes and inoculated with hamster scrapie prions are highly infectious to the hamster but not to the mouse. The same transgenic mouse strain, infected with mouse-derived prions, yields preparations highly infectious for mice but not for hamsters³⁰. In the framework of the protein-only hypothesis this means that hamster PrP^C, but not murine PrP^C, is a suitable substrate for conversion to hamster PrP^{Sc} by hamster prions and vice versa.

It has recently been reported that homozygosity at the polymorphic amino-acid residue 129 of the PrP protein predisposes to acquired¹⁴⁸ and sporadic¹⁴⁹ CJD.

■ A mutated PrP gene can give rise to disease without exogenous infection. The two human prion diseases, CJD and GSS are very rare in the overall population, with a sporadic incidence of $1:10^6$ to $1:10^7$, but also occur as a familial form⁸¹⁻⁸⁴. Hsiao *et al.*⁷⁷ found that in two unrelated GSS families, one in the USA, the other in the UK, the disease is tightly linked to a proline-to-leucine change in codon 102 of one of the alleles of the PrP gene. This mutation was not observed in 100 normal individuals or in 15 individuals suffering from other forms of inherited or sporadic prion diseases. Subsequently, other GSS and CJD families have been identified carrying the 102 mutation or one of a number of other mutations in the PrP gene^{77,85-98}.

Prusiner^{1,21} proposed that the amino-acid substitution allows spontaneous conversion of PrP^C into PrP^{Sc} with a frequency sufficient to allow the disease to be expressed in the lifetime of the individual. Sporadic endogenous CJD and GSS would be attributable to a rare instance of somatic mutation in the PrP gene or of spontaneous conversion of PrP^C into PrP^{Sc}. This hypothesis received strong support from the seminal experiment of Hsiao *et al.*⁹⁹, which showed that mice carrying a murine PrP transgene with the Pro→Leu mutation corresponding to the human GSS mutation at position 102 spontaneously develop a lethal scrapie-like disease. Whether the brains of these animals contain infectious prions has not yet been definitively determined, but it would appear to be the case (S. B. Prusiner, personal communication). These findings support the proposal that a mutated protein, in the absence of exogenous infection, causes scrapie-like disease. But it can be argued that the disease might be caused by a ubiquitous, conventional infectious agent, and that the presence of the mutated PrP greatly increases susceptibility to infection¹⁰⁰.

■ There are many different scrapie prion 'strains'. Dickinson and his collaborators first showed that there are many distinct strains of scrapie prions as judged both by the incubation time in the same mouse strain^{7,69,101-106} and by the pattern of the brain lesions resulting from infection^{32,107-110}. Such strains can be transmitted serially, without changing their properties, in the same mouse strain homozygous for a single PrP genotype^{32,33,111,112}. Therefore it cannot be argued that there are structurally distinct PrP precursor molecules, each of which is specifically convertible into a different PrP^{Sc} strain.

The propagation of different scrapie strains in mice homozygous at their PrP gene is the most difficult finding to explain by the protein only hypothesis, because it implies that an incoming PrP^{Sc} strain can convert a certain PrP precursor into a likeness of itself, and that this can happen for several if not many different strains.

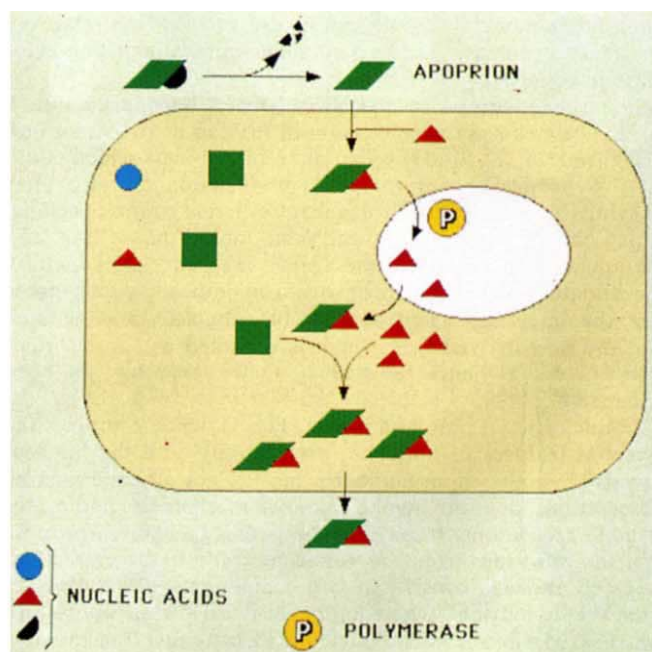
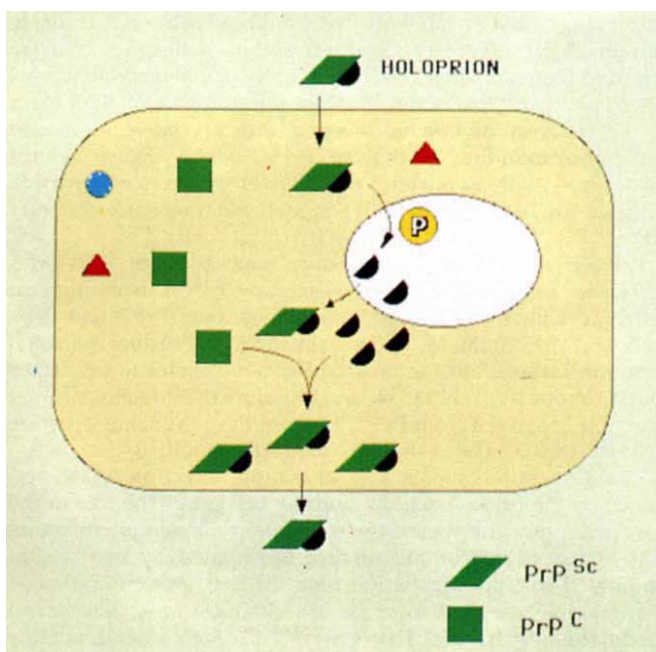


FIG. 1 A model for prion propagation. Left, The holopriion, consisting of PrP^{Sc} (the apopriion) and a nucleic acid (the copriion), invades the cell. Conversion of PrP^C into PrP^{Sc} is mediated by PrP^{Sc} or the holopriion and involves either a chemical modification or a conformational change. The copriion nucleic acid is replicated by a cellular polymerase, a process which is promoted by or dependent on its association with PrP^{Sc}. PrP^{Sc} binds copriion nucleic acid

and reforms holopriions. Right, The holopriion is subjected to nuclease treatment and/or purification, to yield PrP^{Sc}, the apopriion. After penetrating the cell, the apopriion may associate with a potential copriion, namely a cellular nucleic acid to which it has affinity and which can be replicated by a cellular polymerase in the presence of PrP^{Sc}. The resulting holopriion may have phenotypic properties which differ from that of the original holopriion.

■ Prion strains emerge on transfer through different hosts and may mutate. When inocula derived from a natural scrapie source (sheep or goat) are inoculated into different intermediate hosts, different scrapie strains may emerge^{7,111,113}. It is not clear whether these strains preexist and are selected during passaging, or whether they arise *de novo*. It has been proposed that new scrapie strains can arise by mutation. Thus, a scrapie strain biologically cloned in mice, after being passaged in hamsters and then again in mice gave rise to new, different strains as well as yielding the original one^{32,105,114}. These results are difficult to account for by the protein only hypothesis but are formally explained by mutation and selection of a nucleic acid.

Unified theory

The first four propositions above strongly suggest that PrP^{Sc} by itself is a pathogenic agent, whereas the last two argue that the agent comprises of a nucleic acid. These diametrically opposed views can be reconciled if we accept that PrP^{Sc} by itself, the apopron, can initiate the pathogenic process, but that the phenotypic properties which define strain differences are due to the copron, a nucleic acid which is usually associated with PrP^{Sc} but can also be recruited or exchanged in the host cell. This hypothesis explains why scrapie agent preparations appear resistant to ionizing and nonionizing radiation and nucleolytic agents: destruction or removal of nucleic acids should not affect infectivity, but only phenotypic features which were not identified in the inactivation experiments. Conversely, characterization of strains seems to have always been done with crude brain extracts, in which the conjectured phenotype-determining nucleic acid would naturally be present.

The unified theory postulates that PrP^{Sc} is propagated by the conversion of PrP^C or its (perhaps nascent) precursor into a replica of itself, be it by a covalent modification or by a conformational change. This conversion could be caused by PrP^{Sc} (with or without associated copron), essentially as proposed by Prusiner and his colleagues^{3,21,30}. In this connection it is of particular interest to note that wild-type and mutant p53 have different conformations, and that cotranslation of the two proteins forces the wild-type protein into the mutant conformation¹¹⁵. Also, the ATP-dependent assembly of the heat-shock protein hsp60 subunits into the chaperonin 14-mer complex, which is strictly dependent on pre-existing chaperonin complex¹¹⁶, may serve as a model for a catalysed conformational modification of PrP^C (ref. 117). Other examples of self-controlled self-assembly are presented and analysed by Caspar¹¹⁸.

The conjectured copron is most probably a nucleic acid, because it is transmissible and mutable. If it were essential for pathogenesis and propagation and not only involved in modulating the inherent properties of PrP^{Sc}, it would have to be present in all organisms that can be infected by highly purified PrP^{Sc} devoid of nucleic acid or which can develop prion disease without exogenous infection, such as mouse, hamster and man. Nucleic acid capable of serving as a copron may be either encoded by the normal host genome and thus be ubiquitous, or acquired, subsisting as a widely distributed intracellular commensal. Whatever its origin, the copron nucleic acid must be susceptible to direct replication in the cell, because otherwise different scrapie strains could not be propagated unchanged in the same inbred species. The nucleic acid could be an episomal DNA or an RNA; no statement can be made about its size, because all target size determinations have been done on the basis of infectivity and not of phenotypic properties. Because copron-related nucleic acid would be present in normal cells at some level, it may not be easily detected by differential hybridization or by screening a subtraction library^{6,29,45-48}. It has been claimed that the small single-stranded D-loop fragment of mitochondrial DNA is present in preparations enriched for scrapie infectivity⁶⁵, that a small RNA occurs in membrane vesicles from scrapie-infected but not from normal hamster

brain^{119,120} and that 'disease-specific RNA' is synthesized in scrapie-infected mouse brain⁶⁴, however these findings need to be confirmed and their significance documented.

How could a copron introduced into a host cell be amplified? A DNA molecule might be replicated by a nuclear or mitochondrial DNA polymerase. If the copron is an RNA, several possibilities may be envisaged. It has been claimed that normal mammalian cells contain an RNA replicase, however, the data are not compelling^{121,122}. Retrotranscription and integration of the resulting DNA, followed by transcription, is a conceivable mechanism^{66,123}. The replication of an RNA by DNA-dependent RNA polymerase is an intriguing possibility in view of the findings that viroid RNA in plant cells¹²⁴⁻¹²⁶ and most probably hepatitis δ RNA in mammalian cells^{127,128} can be replicated by DNA-dependent RNA polymerase. Moreover, *Escherichia coli* DNA-dependent RNA polymerase¹²⁹ and phage T7 DNA-dependent RNA polymerase^{130,131} can replicate certain RNA species. A specific interaction between such RNA molecules and the polymerase must be postulated, because not any RNA can serve as a template, and an RNA which can be replicated by one kind of DNA-dependent RNA polymerase is not necessarily replicated by another^{130,131}. This suggests that in the cell, RNA variants may compete for the polymerase, leading to selection and amplification of particularly adapted RNA species. If a copron RNA introduced into a cell is particularly suitable for intracellular replication, by virtue of selection in a preceding host, it will outcompete endogenous RNA species and provide a pool of coprons which can associate with the newly formed PrP^{Sc}. If PrP^{Sc} devoid of RNA is introduced into the cell, endogenous RNA may be recruited as copron, and mutation and selection may render it more proficient for its role as copron and template for replication. I postulate that the presence of PrP^{Sc} is required to promote the replication of the copron RNA, just as the presence of the hepatitis delta antigen is required for the replication of the hepatitis δ RNA by a cellular enzyme¹³².

Because the conjectured RNA that can assume the role of a copron may be different in different animals, the passaging of holoprons through different hosts may occasionally lead to an exchange of the nucleic acid and thus to the appearance of different strains. Mutational events at the level of RNA synthesis followed by selection could also lead to the strain shifts reported. In the framework of the protein only hypothesis, the appearance of strains is explained by novel conformations resulting from the interaction of mismatched PrP^C and PrP^{Sc} (S. B. Prusiner, personal communication).

It is interesting to remember in this connection that the phenotypic consequences of many RNA virus infections are modulated by RNA molecules which are derived from the viral RNA or arise from unknown sources, so-called defective interfering RNAs¹³³⁻¹³⁵ and satellite RNAs^{136,137}, respectively. These are replicated in the infected host cells, where they may evolve quite rapidly and adapt to and interfere with the viral RNA replication machinery¹³⁸.

Mice inoculated first with a prion strain with a long incubation time, and then by one with a short incubation time, give rise to the strain injected first^{5,139-143}. This phenomenon has been explained by the 'limited replication site' model, which proposes that a rate-limiting site or process is fully occupied by the first strain to invade the host, so that the second strain is deprived of a critical step in replication. In terms of the unified theory, the nucleic acid introduced with the first scrapie strain would be amplified extensively by the time the second strain is introduced, and the nucleic acid introduced with the second strain would not be able to compete with that of the first.

Testable predictions

The unified theory makes several testable predictions, the first of which may serve to falsify it.

■ Strain-specific properties of prions are lost after removal of nucleic acids. If preparations of two distinct scrapie strains are

extensively purified and the nucleic acids effectively eliminated or destroyed, then the phenotypic properties of the two strains, such as incubation or lesion pattern, should revert to the same type in the same host strain.

■ The strain-specific properties of a prion can be changed by replacing the nucleic acid associated with it by that derived from another strain. If PrP^{Sc} from a scrapie strain A, purified free of nucleic acid, is mixed with the deproteinized nucleic acid fraction of brain extract from an animal infected with a scrapie strain B, the resulting preparation should give rise to strain B when inoculated into animals. The reciprocal results should be obtained in the converse experiment.

■ PrP^{Sc} should specifically bind certain kinds of nucleic acids. Purified PrP^{Sc} preparations devoid of nucleic acids should preferentially bind some species of radioactive nucleic acids from scrapie-infected brain or partially purified PrP^{Sc} preparations. Binding requirements could be analysed by published methods^{144,145}.

Discussion

The unified theory incorporates most features of the protein only hypothesis and therefore adopts the explanations offered by the latter for the species barrier phenomenon and for the origin of sporadic and genetically determined prion diseases. The theory accepts the premise of the virino hypothesis that a nucleic acid is responsible for some phenotypic features and is replicated in the cell, but it denies that this nucleic acid is required for infectivity and that it represents an independent, agent-specific genome.

I propose the unified theory to reconcile apparently contradictory conclusions regarding the requirement for a nucleic acid, invoked to explain the existence of scrapie strains, and alternatively the apparent absence of nucleic acid in prions. But the premises on which these conclusions are based are neither conclusive nor undisputed. Despite the fact that it seems unlikely to many researchers, PrP^{Sc} derived from the same precursor might indeed assume many different conformations, making the unified theory unnecessary. A small nucleic acid required for infectivity may yet be found and, less likely, the data interpreted as proof for the existence of strains may be explained otherwise. The suggestion that PrP^{Sc} is a conformational isomer of PrP^C is based on the failure to find a chemical difference between the two forms but, as mentioned in the second proposition, the infectious entity, even if it is derived from PrP^C, need not be PrP^{Sc} as defined by its physical properties³, and could be chemically different either in regard to its primary structure or to posttranslational modifications. Mechanisms which might generate variants of PrP differing in their amino-acid sequence include editing at the messenger RNA level¹⁴⁶ or specific mistranslation¹⁴⁷; to explain stable scrapie strains, these variations would have to be steered specifically by the individual PrP species, for which a novel mechanism has to be invoked.

In closing, I would like to stress that the theory outlined in this article is based entirely on biochemical reactions for which precedents are known, even though the overall combination of these steps is unusual. But then so is the disease. □

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ACKNOWLEDGEMENTS. I thank S. Prusiner for many productive discussions and insightful critique, and P. Sharp, M. Aebi, H. Büeler and M. Fischer for reading the manuscript and for discussion.

Deep structure of the northeastern Japan arc and its relationship to seismic and volcanic activity

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Evidence for possible deep-seated magmatic activity beneath northeastern Japan can be obtained from seismic observations. Tomographic inversions of P-wave velocity data show low-velocity zones distributed in the crust and upper mantle beneath active volcanoes. In or around these zones, anomalously low-frequency micro-earthquakes, perhaps caused by magmatic activity, are found at depths of 25–40 km; distinct S-wave reflectors, corresponding to the upper surfaces of magma bodies, are found at depths of 10–18 km. Large crustal earthquakes also occur around the low-velocity zones. These observations reveal the distribution of magma reservoirs at depths and elucidate its relation with shallow seismicity beneath volcanic arcs.

NORTHEASTERN Japan is located at one of the most typical subduction zones in the world, where the oceanic Pacific plate plunges downward into the mantle under the continental plate. Seismic activity is extremely high along the main thrust zone beneath the landward slope of the Japan trench. This shallow seismicity (shallower than 50–60 km) is characterized by low-angle thrust faulting¹, which reflects directly the relative movements of the two converging plates and the downward motion of the subducting plate. The shallow seismicity along the main thrust zone merges continuously with the deep seismic zone which dips beneath northeastern Japan. The deep seismic zone, originating not at the boundary between the two converging plates but within the subducted Pacific plate, is composed of two thin planes, parallel to each other and 30–40 km apart². The upper seismic plane is characterized by down-dip compression, whereas the lower plane by down-dip extension². Formation of the double seismic zone can be explained at least qualitatively by the unbending of subducted oceanic plate³.

In addition to these seismicities, shallow earthquakes confined mainly to the upper crust⁴ occur beneath the inland area. Although the activity of these crustal events is less severe than that along the main thrust zone, historically severe damage has been inflicted by many crustal earthquakes because they are close to populated areas. Many active volcanoes are distributed in the land area, mainly along the volcanic front which runs

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through the middle of the land area nearly parallel to the trench axis. Eruptions of these active volcanoes, although not frequent, have been another source of natural disaster in this country.

Here, we present a precise three-dimensional seismic velocity structure of the crust and upper mantle beneath this volcanic arc obtained by using data acquired through microearthquake observations. Tomographic images of the estimated three-dimensional P-wave velocity structure show low-velocity (low-V) zones, which are continuously distributed from the upper mantle to the upper crust beneath active volcanoes. Exceptionally deep microearthquakes (25–40 km) of anomalously low frequency (LF) are detected at nine locations around the low-V zones beneath active volcanoes, suggesting a close relation with the magmatic activity in this depth range. At shallower depths (10–18 km) in or around the low-V zones, we find distinct reflectors of S-waves. The locations of reflection points and the calculated reflection coefficients suggest that the reflectors depict the upper surfaces of magma bodies in the mid-crust.

P-wave velocity structure

Tomographic studies beneath the Japan islands have been made by several researchers. Hirahara *et al.*⁵ investigated the P-wave velocity structure beneath the central part of Japan, finding low-V zones in the wedge portion of the upper mantle above the high-V Philippine Sea and Pacific plates subducting beneath central Japan. Hasemi *et al.*⁶ and Obara *et al.*⁷ investigated the structure beneath the central part of northeastern Japan, and found low-V zones in the crust and upper mantle beneath active volcanoes. These results strongly suggest that the low-V zones are closely related to arc volcanism and indicate the importance of tomographic studies for understanding arc volcanism, although they involve the problems described below.

Seismic velocity discontinuities (SVDs), such as the Moho and Conrad discontinuities and the upper boundary of the subducted oceanic plate, are known to exist in the crust and upper mantle beneath the northeastern Japan arc and to have large undulations^{8–11}. Most tomographic studies made so far, however, do not consider the effect of the complicated shapes of the SVDs, or even their existence. Thus estimated tomographic images include not only velocity variations but also the effect of SVDs, especially in their vicinity. Another problem involved in most of the conventional tomographic studies is that three-dimensional ray tracing is not used to calculate seismic ray paths and travel times. In very heterogeneous regions such as subduction zones, ray paths calculated from a simple one-dimensional velocity model deviate considerably from the real paths for seismic waves with large hypocentral distances; this seriously distorts the final tomographic images.

A new method of seismic tomography has been developed to solve these problems¹². This method is applicable to a general velocity structure with many SVDs of complicated shapes in the modelling space and with three-dimensional variations in the seismic velocity in each layer bounded by these discontinuities. Three-dimensional grid nets are arranged in every layer individually, and the velocity at each grid point is taken to be an unknown parameter. A velocity at any point in the modelling space is calculated by linearly interpolating the velocities at eight surrounding grid points. Velocity parameter derivatives are calculated by using a linear interpolation function expressing the velocity field. An efficient three-dimensional ray-tracing algorithm has been developed which iteratively uses the pseudo-bending technique¹³ and Snell's law. This algorithm calculates ray paths and travel times rapidly and accurately for seismic waves in a complicated velocity structure. The algorithm developed here can include in the inversion the arrival-time data of later phases, such as reflected or converted waves at the SVDs, which greatly improve the ray coverage in modelling space. A conjugate gradient solver, the LSQR algorithm¹⁴, is used to solve the extremely large and sparse system of observation equations

arising from the inversion problem. Hypocentre and velocity parameters are determined simultaneously. Because of the LSQR algorithm, it is unnecessary either to set up the normal equation or to use the parameter separation technique, which are very time-consuming and formidable when the number of unknown parameters is large.

High-resolution P-wave tomographic images of the crust and upper mantle beneath the Japan islands are obtained by applying the method to arrival-time data observed by the seismic networks of several national universities in Japan. In addition to first P- and S-wave arrivals, later arrivals of SP waves converted at the Moho discontinuity, and PS and SP waves converted at the upper boundary of the subducted Pacific plate, are read from seismograms observed by the seismic network of Tohoku University and are included in the inversion. We selected 512 shallow events and 688 intermediate-depth and deep events which are distributed uniformly in the upper crust and in the depth range of 40–650 km along the Wadati–Benioff zones corresponding to the subducted Philippine Sea and Pacific plates. In total, 50,919 data points corresponding to arrival times observed at 208 seismic stations distributed in the Japan Islands are collected from the 1,200 events.

The medium under study is divided into four layers by the Conrad and Moho discontinuities and the upper boundary of the subducted Pacific plate, these four layers corresponding to the upper crust, the lower crust, the wedge mantle and the mantle beneath the upper boundary of the Pacific plate. The depth distributions of these SVDs are fixed and expressed by continuous functions of spatial locations so as to coincide with the results of previous studies^{10,15,16}. We arrange 1, 1, 16 and 16 layers of grid nets in the upper crust, the lower crust, the wedge mantle and the mantle beneath the upper plate boundary, respectively. The separation between grid points is 25–30 km in both vertical and horizontal directions. Three iterations are carried out in the nonlinear inversion, as the difference in r.m.s. residual of the inverted solutions between the second and third iterations is sufficiently small. In total, a 48% variance reduction is achieved. Checkerboard resolution tests^{17,18} with various wavelengths of velocity change are made to evaluate the resolution of the tomographic images obtained. The original patterns of the checkerboards are well reconstructed for the study area. Reconstructed amplitudes of velocity anomalies are more than 80% of the original amplitudes for most grids shallower than ~200 km, and 60–70% for those deeper than that. The uncertainty of the inverted velocity perturbations is estimated to be ~0.5% for grids shallower than ~200 km and less than 1% for deeper grids. These indicate that a meaningful solution is accurately obtained.

We have updated the tomographic images of northeastern Japan by Hasemi *et al.*⁶ and Obara *et al.*⁷ by covering a wider area and by using higher resolution. Figure 1 shows the estimated fractional P-wave velocity perturbations at a depth of 40 km beneath northeastern Japan. As seen from this figure, most active volcanoes are located in the low-V zones. Figure 2a shows an east–west vertical cross-section. The low-V zone located just beneath active volcanoes in the crust and the uppermost mantle dips to the west in the wedge mantle and extends to a depth of ~150 km. This feature is common to all the low-V zones located in the crust and the upper mantle beneath northeastern Japan. Although in the cross-section shown in Fig. 2a, the low-V zone seems to be split into two parts, one shallower (~50 km), the other deeper (~100 km), the other low-V zones beneath active volcanoes continuously extend to a depth of ~150 km; one example is shown in Fig. 2b. A high-V zone corresponding to the subducted Pacific plate is clearly seen. The thickness of the high-V zone is estimated to be 80–90 km. This agrees with the recent study by Umino *et al.*¹⁹ who detected a later phase reflected from the bottom of the high-V Pacific plate beneath northeastern Japan and estimated its thickness to be ~85 km from arrival-time analysis. Figure 2a also shows that the double

seismic zone is located in the upper half of the high-V Pacific plate.

Low-frequency microearthquakes

More than 98% of shallow microearthquakes in the land area of northeastern Japan occur in the upper 15 km of the crust. The cut-off depth for these shallow events can be interpreted as a brittle-ductile transition or a stick-slip to stable-sliding transition due to increasing temperature with depth^{20–22}. The upper 15 km of the crust forms a brittle seismogenic zone. A systematic investigation of shallow inland microearthquakes reveals however, that exceptionally deep events do occur (although they are rare) at nine locations in northeastern Japan in the depth range 25–40 km, which is well below the base of the brittle seismogenic zone. The characteristics of these anomalous events are as follows: their focal depths are anomalously deep (25–40 km); the predominant frequencies of both P- and S-waves are extremely low (1.5–3.5 Hz); the maximum magnitude is at most 2.5; and they occur around active volcanoes or around the low-V zones.

Examples of seismograms of these anomalously deep events are shown in Fig. 3a. Figure 3b represents an event whose epicentre is close to the event shown in a, but which has a normal focal depth (10 km). What is obvious on comparing the two seismograms is that this anomalously deep event has extremely low predominant frequencies both for P- and S-waves; this was found to apply to all the events at depths 25–40 km found at the nine locations. Intermediate-depth earthquakes at depths 70–150 km, occurring in the double seismic zone just under these LF events, again have normal predominant frequencies for P- and S-waves. This indicates that the low predominant frequencies of these anomalous events are not due to the propagation path but to the source.

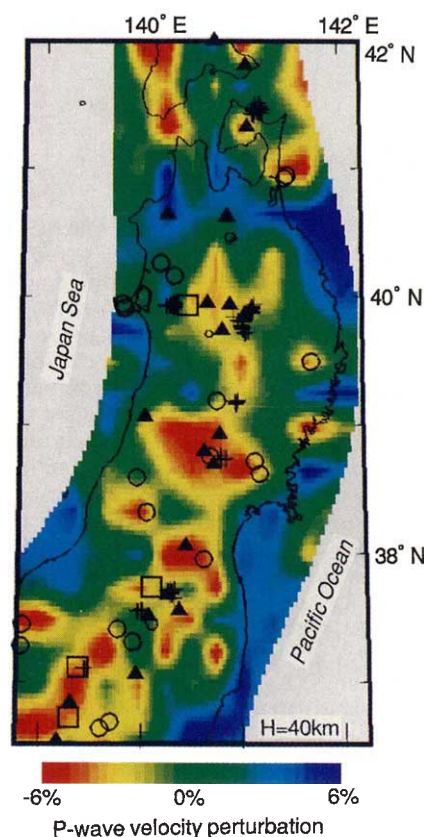
The hypocentres of the LF events are indicated by crosses in Fig. 1 and by red circles in Fig. 2a. We can see from these figures that LF events are located approximately beneath active

volcanoes or around low-V zones. The magnitudes of these LF events are not large enough to determine a unique focal mechanism solution by using the initial motions of the P-wave. It seems that the distributions of P-wave initial motions on the focal sphere for two relatively large events cannot be explained by a double-couple mechanism, although the polarity data are too sparse and their coverage on the focal sphere are too poor to insist on non-double-couple mechanisms for these two events. Nevertheless the closeness to active volcanoes and other features described above strongly suggest that these anomalous events are caused by magmatic activity in the depth range 25–40 km.

Distinct S-wave reflectors

We have detected distinct S-wave reflectors in the mid-crust at two locations in northeastern Japan: one in northern Akita prefecture and the other in southeastern Yamagata prefecture. Figure 4 gives examples of seismograms showing reflected S-waves from one of these two reflectors. We can see a sharp impulsive later phase following the direct S-wave at all three stations. The main features of this unusual phase are as follows: (1) the phase is most clearly defined on instruments measuring the horizontal component; (2) the predominant frequency of the phase is nearly the same as that of direct S-wave; and (3) the amplitude of this phase is very large, and in some cases even larger than that of the direct S-wave. Arrival-time analysis of this phase together with the above-mentioned features indicate that this unusual phase is an S-wave reflected from a sharp velocity discontinuity in the mid-crust (S×S phase), similar to that first detected and identified beneath the Rio Grande rift near Socorro, New Mexico^{23,24}. A reflected and S-to-P converted phase (S×P phase) at the same discontinuity can be also found at station NIB. This S×P phase is most clearly seen in the vertical component, although its amplitude is not very large. The large amplitude of the S×S phase and the ratio of S×P to S×S amplitudes are explained by a large velocity contrast across

FIG. 1 Fractional P-wave velocity perturbation (in %) at a depth of 40 km beneath northeastern Japan. The velocity perturbation is from the mean value of estimated velocities at this depth. Red and blue colours, low and high velocities, respectively. Solid triangles, crosses, open squares and open circles denote the locations of active volcanoes, low-frequency microearthquakes at depths of 25–40 km, distinct S-wave reflectors in the mid-crust and large crustal earthquakes, respectively.



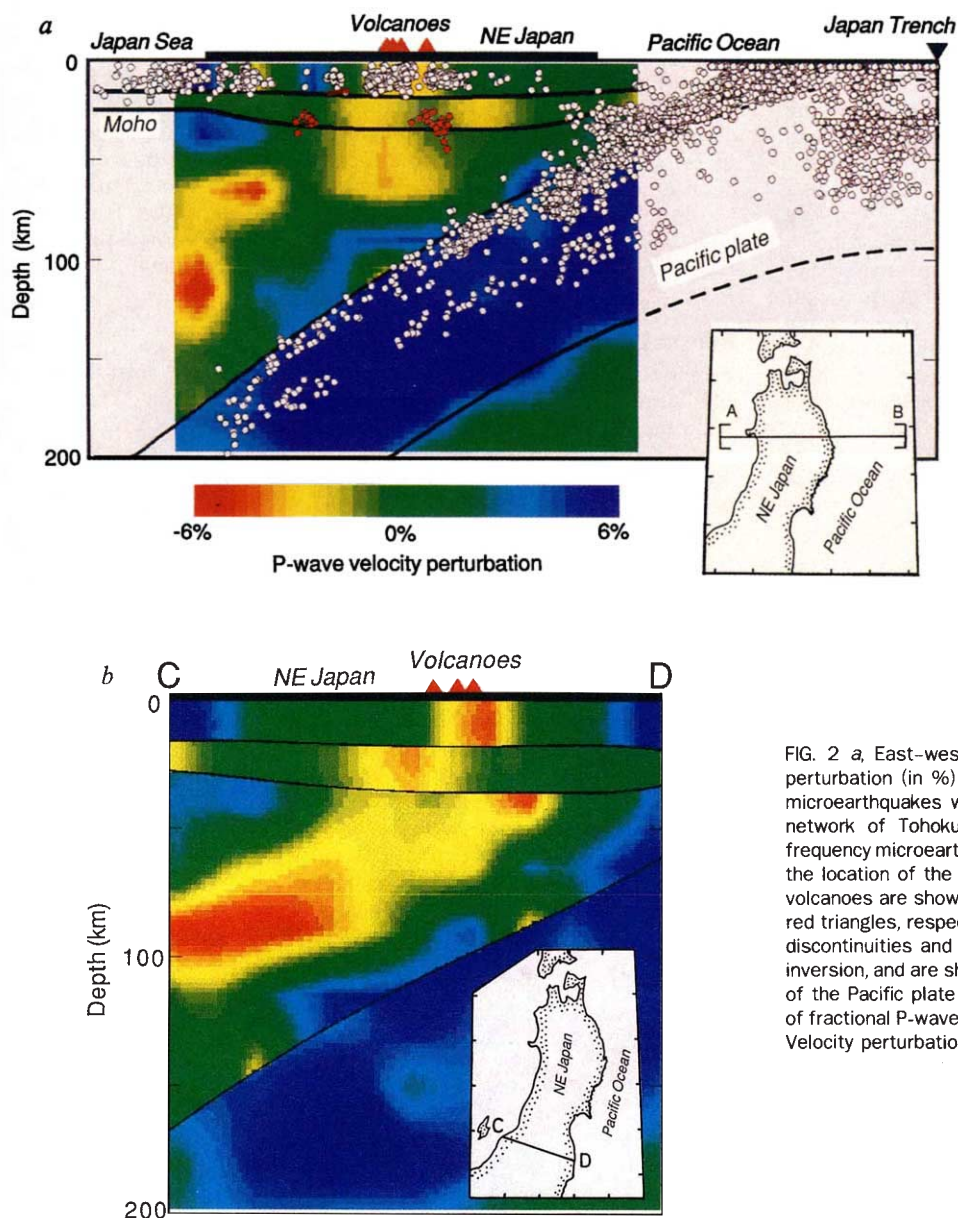


FIG. 2 *a*, East-west vertical cross-section of fractional P-wave velocity perturbation (in %) along the line AB in the inset map. Open circles are microearthquakes within a 60-km width along AB located by the seismic network of Tohoku University in 1987–1990. Red circles denote low-frequency microearthquakes located in 1975–1990, and a red line represents the location of the mid-crustal S-wave reflector. The land area and active volcanoes are shown at the top of the figure by the bold horizontal line and red triangles, respectively. The depth distributions of the Conrad and Moho discontinuities and the top of the subducted Pacific plate are fixed in the inversion, and are shown by bold lines. The estimated location of the bottom of the Pacific plate is also shown by a bold line. *b*, Vertical cross-section of fractional P-wave velocity perturbation along the line CD in the inset map. Velocity perturbation is shown by the colour scale as in *a*.

a discontinuity underlain by a very-low-rigidity material, such as a magma body, as pointed out by Sanford *et al.*²³.

We try to map this velocity discontinuity by analysing the arrival times of the S×S phase observed at the four stations shown in Fig. 5. By assuming the velocity discontinuity to be a single flat plane, we can locate the reflection point for each of the observed S×S phases from the S×S arrival-time data in combination with accurately determined hypocentre coordinates. The dip of the discontinuity is corrected in the same way as seismic migration. Estimated locations of the reflection points are indicated by open circles in Fig. 5. They form a gently inclined flat plane with scatter of ± 1 km, showing that the original assumption that the velocity discontinuity is a simple flat plane is adequate. The amount of scatter (± 1 km) corresponds to the accuracy of the hypocentre locations and that of the S×S phase readings. As presently mapped, this unusual S-wave reflector is distributed over an area of 15×15 km² at depths of 12–17 km beneath a Quaternary volcano. The reflector slopes at an angle of $\sim 15^\circ$, becoming shallower towards the ESE, in which direction more active volcanoes are densely distributed. Beneath this reflector, a group of LF microearthquakes occur at depths 27–37 km.

Distinct S-wave reflectors, similar to that described above,

have also been found in two other locations in northeastern Japan^{25–27}. The locations of the reflectors detected here and those detected in previous work are plotted by large squares in Fig. 1. As is obvious from this figure, all the reflectors are located near active volcanoes and/or in or around the low-V zones. LF microearthquakes occur at greater depths beneath the reflectors. These observations and the features of S×S phase described above support the evidence that the reflectors are the upper surfaces of magma bodies in the mid-crust. Detection of the reflector requires a favourable geometrical relation between source, receiver and reflector. Even if a distinct reflector actually exists, we cannot detect it without both earthquakes and stations just above the reflector. This suggests that many other reflectors may exist at different locations in northeastern Japan, because seismicity is not homogeneous in space and seismic stations are not densely distributed. The velocity decrease of several percent in the low-V zones estimated by the present tomographic study also suggests the existence of partially molten materials in the mid-crust and in the upper mantle.

No reliable information has been obtained to determine the bottom or the thickness of the magma body hypothesized here. Direct S-waves at station MOR from intermediate-depth earthquakes right under it, which pass through the estimated

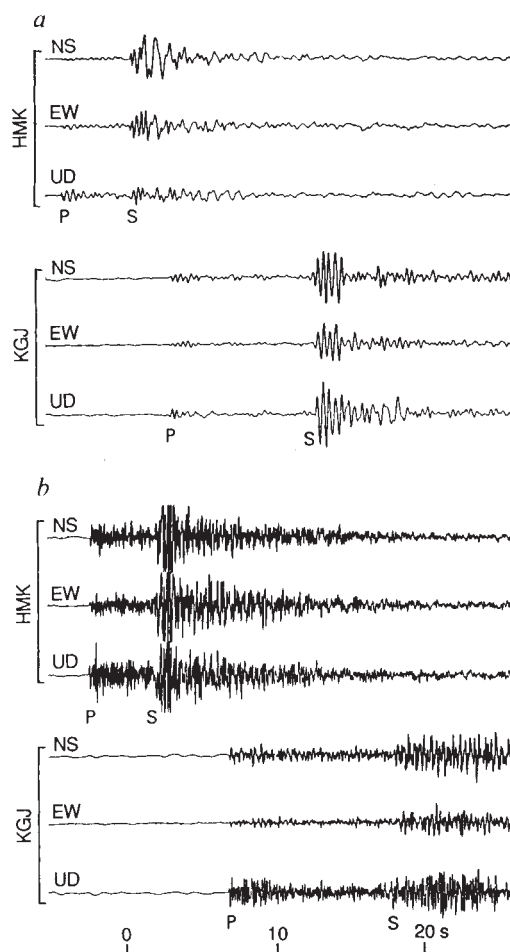


FIG. 3 Examples of short-period (1 s) three-component seismograms of *a*, an unusually deep (32 km) low-frequency microearthquake and *b*, a normal depth (10 km) event, which occurred near Iwate volcano. Magnitudes of the two events are 1.6 and 2.8, respectively.

magma body in the mid-crust, have spectra no different from those at other stations. In other words, S-waves passing through the magma body are not significantly attenuated. This fact suggests that the magma body is very thin, probably between several hundred metres and a few kilometres, similar to that detected beneath the Rio Grande rift in the vicinity of Socorro, New Mexico²⁴.

Deep structure

In the land area of northeastern Japan, shallow crustal seismicity is confined to the upper 15 km of the crust, with the exception of a few deep LF microearthquakes as already shown. If examined in more detail, the greatest depth of this shallow seismicity, which is sharply delimited, varies with the location. We have found that, in the central part of northeastern Japan where seismic stations are densely distributed, the cut-off depth for the shallow seismicity locally elevated becomes shallower at some places near active volcanoes; this can be explained by the local elevation of the brittle-ductile transition zone because of the higher temperature in the crust in those areas.

The crust and upper-mantle structure beneath northeastern Japan can be inferred as schematically illustrated in Fig. 6, by combining all the observations presently obtained: low-V zones in the crust and upper mantle, LF microearthquakes at depths 25–40 km, S-wave reflectors in the mid-crust and local elevation of the base of the seismogenic zone beneath active volcanoes. Low-V zones in the uppermost mantle are the manifestation of mantle diapirs which form the roots of arc volcanoes at depth. Magmatic activity of the mantle diapir may cause the occurrence of LF microearthquakes around it, although they occur very infrequently. In the mid-crust, molten materials rising from

below show themselves as distinct S-wave reflectors which are presumed to be the tops of very thin magma bodies.

Shimamoto²⁸ estimated the spatial distribution of mechanical strength in the crust and upper mantle beneath the northeastern Japan arc from the rheological properties of rocks. Temperature increases relatively rapidly with depth beneath this volcanic arc; this results in a very thin brittle seismogenic zone in the crust. Its thickness is estimated to be ~15–20 km, corresponding to the actual cut-off depth of 15 km for shallow crustal seismicity. The lower portion of the crust and the wedge mantle, below the base of the brittle seismogenic zone, are governed by creep or flow, being weak and incapable of supporting much stress. Consequently horizontal compressional stress in the direction of relative plate motion, acting on the crust and the upper mantle, is supported mostly by the upper 15 km of the crust, the relatively strong seismogenic zone.

In this situation, the stress will concentrate in or around the places where the base of the seismogenic zone is locally elevated, and finally would cause shallow crustal earthquakes. In places where the base of the seismogenic zone is extremely shallow, such as in the vicinity of active volcanoes, the stress will be released by creep or flow, and thus shallow crustal earthquakes will occur not in but around such areas. Local elevation of the base of the seismogenic zone may be due to molten materials having ascended from greater depths. Shallow crustal earthquakes are not homogeneously distributed in space over the land area beneath this volcanic arc, but are concentrated in particular locations. We suggest the mechanism described above is the main cause for this concentration of crustal events.

The depth to the brittle-ductile transition zone is prescribed principally by the thermal structure in the mid-crust. At present,

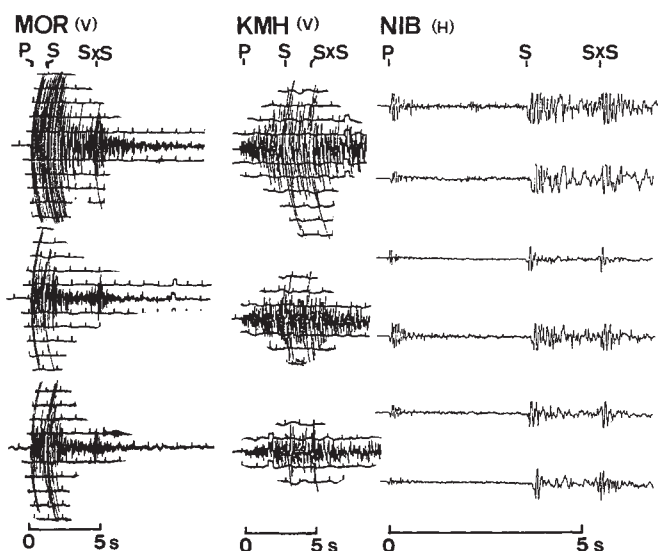


FIG. 4 Examples of short-period seismograms of shallow microearthquakes that occurred near Mt Moriyoshi, showing clear later arrivals of S-waves which are presumed to be reflected S-waves ($S \times S$) at the top of magma body. Arrival times of direct P- and S-waves are denoted by P and S on the top, and V and H in parentheses indicate vertical and horizontal components. Locations of seismic stations MOR, KMH and NIB are shown by crosses in Fig. 5, where the focal area of these events is indicated by the shaded area.

the spatial distribution of temperature in the mid-crust is not precisely known. As a substitute for the temperature distribution, we use the P-wave velocity distribution at a depth of 40 km; this is the depth at which the most accurate velocity distribution is obtained, according to the resolution analyses. If low velocity corresponds to high temperature, we can consider Fig. 1 as a map showing a first approximation to the temperature distribution. Thus the base of the seismogenic zone might be locally elevated in the low-V zones in Fig. 1 because of the higher temperature, causing stress concentration around these zones under the tectonic stress field of horizontal compression. In Fig. 1, open circles indicate large crustal earthquakes that occurred since 1931 (ref. 29). It is seen from this figure that many of these occurred around the low-V zones. Crustal earthquakes with smaller magnitudes have a similar tendency, which is partly seen on the vertical cross-section in Fig. 2a; many events in the upper crust beneath the land area are concentrated in or around the low-V zones. The low-V zone corresponding to high seismicity is also found in the Mount St Helens region and is presumed to be the lineation of a crustal weak zone³⁰. Of course, the P-wave velocity distribution obtained in this study is not an adequate approximation for the temperature distribution in the crust, which is the information we truly need to know precisely. Although Fig. 1 seems partly to support our interpretation,

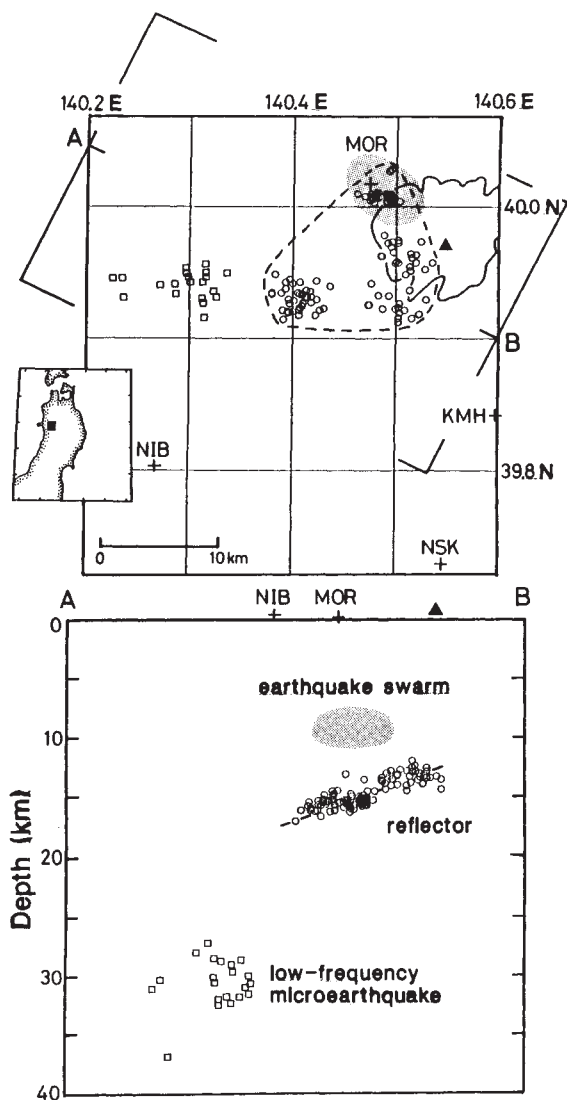


FIG. 5 Locations of deep low-frequency microearthquakes, the mid-crustal S-wave reflector and an earthquake swarm beneath Mt Moriyoshi, northern Akita Prefecture. a, Epicentres of low-frequency events (open squares), earthquake swarm area (shaded area) and S-wave reflection points (open circles). b, Vertical cross-section along AB in a. Solid triangle and crosses show the locations of Mt Moriyoshi (a Quaternary volcano) and observation stations, respectively.

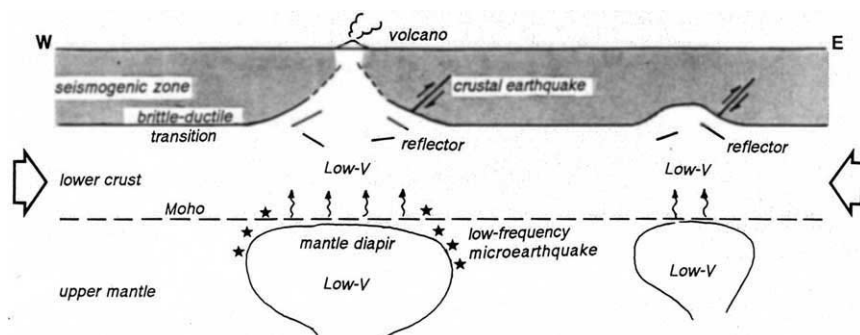


FIG. 6 Schematic east-west cross-section of the crust and upper-mantle structure beneath north-eastern Japan. The depth to the brittle-ductile transition zone in the crust locally becomes shallow, perhaps because of ascending magma, which will cause stress concentrations around regions where the base of the brittle seismogenic zone is locally elevated.

further investigation of the detailed structure of the crust and upper mantle is necessary for confirmation.

We can say, however, that the observations obtained here from seismic data indicate the deep structure of volcanoes beneath the northeastern Japan arc. Deep roots of volcanoes

are seen from the clear low-V zones of P-wave in the crust and the wedge mantle beneath active volcanoes, the anomalously deep (25–40 km) LF microearthquakes around the low-V zones, and the distinct mid-crustal reflectors of S-wave in or around the low-V zones. □

Received 19 March; accepted 23 July 1991.

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ACKNOWLEDGEMENTS. We thank A. T. Linde at DTM for critically reading the manuscript. In the tomographic study we used arrival time data from the Japan University Network Earthquake Catalogue published by Earthquake Research Institute, University of Tokyo.

Cloning of a human gene encoding the general transcription initiation factor IIB

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Transcription factor IIB (TFIIB) has a central role in transcription of class II genes. The purification of the human TFIIB protein and isolation of a complementary DNA encoding TFIIB activity is reported here. The sequence of TFIIB, which seems to be encoded by a single gene, contains a repeated motif, in addition to a motif with similarity to the prokaryotic σ -factors. The recombinant protein expressed in bacteria substituted for all the functions attributed to the human TFIIB protein.

INITIATION of transcription at class II promoters requires, in addition to RNA polymerase II, two functionally distinct classes of transcription factors. One such class, the general transcription factors, operates through common promoter elements (TATA and/or initiator motifs) and is required for transcription of all class II genes analysed. At least seven different protein factors: TFIIA, -IIB, -IID, -IIE, -IIF, -IIG and -IIH comprise the general transcription factors^{1,2,3}. Binding of TFIID to the TATA motif seems to be the first step in the formation of a transcription-competent complex, providing a recognition site for the association of the other general transcription factors and RNA polymerase II (refs 4, 5, 50, 51). The gene encoding human TFIID has been isolated^{6–8}. The activity is in a single polypeptide of relative molecular mass about 37,000 (M_r , 37K). But TFIID isolated from HeLa cells purifies as a large complex (larger than 150K), presumably because TFIID is associated with other undefined proteins⁹. Kinetic analyses and complex formation

studies have suggested that TFIIA acts at an early step of the transcription cycle^{10–12}, presumably by facilitating binding of TFIID to the TATA box, thereby generating the TFIID–IIA (DA) complex^{4,5}. TFIIA has been purified to apparent homogeneity using affinity chromatography on columns containing immobilized TFIID protein (P. Cortes & D.R., manuscript in preparation). TFIIB associates with the DA complex resulting in the formation of the TFIID–IIA–IIB (DAB) complex⁵. The DAB complex is then recognized by RNA polymerase II. The association of the polymerase requires TFIIF, a factor that interacts with RNA polymerase II (refs 13, 14), and is composed of two subunits¹⁵, the smallest of which contains sequence similarity to the prokaryotic factor, sigma 70 (ref. 16). The association of TFIIF/RNA polymerase II with the DAB complex allows recognition by TFIIE, TFIIG and TFIIH which bind to the complex (O. Flores *et al.*, manuscript in preparation). TFIIE has been purified to apparent homogeneity and copurifies with polypeptides of 34 and 56K (refs 17, 18). TFIIG and TFIIH are two newly identified general transcription factors that have been partially purified (ref. 3, O. Flores *et al.*, manuscript in preparation).

The second class of transcription factors recognizes specific DNA sequences present in promoters¹⁹. These factors, the specific transcription factors, regulate levels of expression and in some cases mediate the response to a specific stimulus. The molecular mechanism(s) by which the specific transcription factors affect transcription is unknown. But there is evidence to suggest that these factors communicate directly (through protein–protein interactions) or indirectly (DNA mediated) with the general transcription factors. Recent studies have suggested that the activating domain of VP16 can directly interact with TFIID (ref. 20). It has also been suggested that the VP16 activator can facilitate the association of TFIIB with the preinitiation complex²¹. Towards our goal of unequivocally determining

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the roles of the general transcription factors during transcription-complex formation we have purified TFIIB and isolated, using 'reverse genetics', a cDNA clone encoding this activity.

Purification of TFIIB

TFIIB was purified to apparent homogeneity using a functional transcription assay reconstituted with the general transcription factors, RNA polymerase II and the major late promoter of adenovirus (Ad-MLP) as template, as described in Fig. 1. The polypeptide compositions of the different chromatographic steps are shown in Fig. 1a. The last step of the procedure, chromatography over a phenyl superose column, was enriched in a polypeptide with an apparent M_r of 33K.

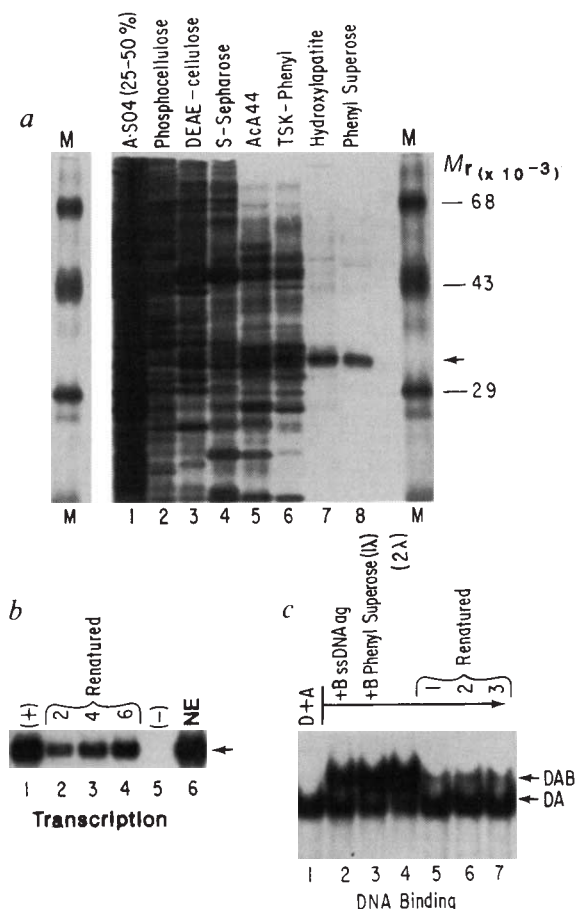
To analyse whether the isolated 33K protein contained TFIIB activity, the polypeptide was isolated by reversed phase chromatography and after renaturation, assayed for activities known to be associated with TFIIB (that is, transcription and association with the preinitiation-complex). The addition of increasing amounts of renatured protein to a transcription assay reconstituted with highly purified transcription factors resulted in activity (Fig. 1b, lanes 2–4). The levels of activity observed with the renatured protein were about 40% of those observed with native TFIIB. TFIIB activity associates with the DA complex to generate the DAB complex, both of which are intermediates during formation of a transcription-competent complex^{4,5}. Addition of increasing amounts of renatured 33K protein to

DNA binding reaction mixtures containing TFIIA, TFIID and a labelled DNA fragment including the Ad-MLP, resulted in the production of a DNA protein complex migrating slower than the complex formed in the absence of TFIIB (DAB, Fig. 1c, compare lanes 5–7 with 1). The DNA-protein complex formed with the renatured protein comigrated on the native gel with the complex formed with the unrenatured protein (compare lanes 5–7 with 3 and 4) and with a control TFIIB protein (lane 2). Thus TFIIB activity is contained in a single polypeptide of 33K. This polypeptide is necessary for transcription and binds to the DA complex.

Molecular cloning of TFIIB protein

Purified human TFIIB protein was digested with the endoproteinase LysC and the resulting peptides were isolated by reverse phase chromatography. Four peptides were sequenced (Fig. 2, a–d) and degenerative oligonucleotides (Fig. 2, 1–4) based on the amino-acid sequence from peptides 'a' and 'd' were synthesized. Sequence amplification using a HeLa cDNA library mediated by the polymerase chain reaction (PCR) and primers '1' and '4' yielded a single product of about 500 nucleotides. A HeLa cell cDNA library was screened with an oligonucleotide derived from the PCR product (Fig. 2, oligo 5). The complete nucleotide sequence of the longest cDNA clone isolated is shown in Fig. 2. The nucleotide sequence of human TFIIB predicts a long open reading frame encoding a poly-

FIG. 1 Purification of TFIIB. a, Silver staining of a polyacrylamide-SDS gel containing aliquots (equal transcription units) of fractions derived from the different steps of the purification of TFIIB, as indicated on the top of the panel. b, The 33K polypeptide was isolated by reverse-phase chromatography (RPC) and the renatured protein assayed in a transcription assay reconstituted with purified general transcription factors (A, D–H), RNA polymerase II and a plasmid DNA containing the Ad-MLP as previously described⁵. +, reactions performed in the presence (phenyl Superose fraction, 15 ng) and absence (–) of TFIIB. NE, reaction mixtures reconstituted with HeLa cell nuclear extract (9 µg protein). c, TFIIB activity in the renatured protein was measured for binding to the DA complex using a gel mobility shift assay containing a 3'-end labelled DNA fragment (Ad-MLP from –45 to +18) as previously described⁵. The fraction used in lane 2 (ssDNAag) was derived using an alternative protocol to isolate TFIIB (ref. 5). **METHODS.** TFIIB was purified from S100 of HeLa cells, a side fraction in the preparation of nuclear extract³¹. S100 (10 l; 80 g protein derived from about 2×10^{12} cells) were used in the purification of TFIIB. The S100 was thawed on ice and proteins precipitated with solid $(\text{NH}_4)_2\text{SO}_4$, which was added slowly to 25% saturation. The protein solution was neutralized by the addition of 1 µl 1 M NaOH per g $(\text{NH}_4)_2\text{SO}_4$. The precipitated proteins were collected by centrifugation at 8×10^3 r.p.m. for 1 h using a Sorvall GSA rotor. Proteins in the supernatant were further precipitated with solid $(\text{NH}_4)_2\text{SO}_4$ to 50% saturation. The precipitated proteins were collected as described above and resuspended in buffer C (20 mM Tris–HCl buffer, pH 7.9, 4 °C, 20% (v/v) glycerol, 10 mM β-mercaptoethanol, 0.1 mM EDTA) to 500 ml. The solution was dialysed overnight against 8 l buffer C containing 0.1 M KCl (fraction I). Debris present in fraction I was removed by centrifugation at 25×10^3 r.p.m. for 25 min using a Sorvall T647.5 rotor. The solution was adjusted to a conductivity equal to 0.1 M KCl with buffer C and proteins (37 g) were loaded onto a phosphocellulose column (10 × 45 cm, 10 mg protein per ml resin). Fractionation on phosphocellulose was done as previously described¹⁰. Proteins eluting between 0.3 and 0.5 M KCl were pooled and dialysed overnight against 8 l buffer C containing 0.1 M KCl. Proteins (1.7 g) were then loaded onto a DEAE-cellulose column (5 × 35 cm, 5 mg protein per ml resin) that was equilibrated with buffer C containing 0.1 M KCl. TFIIB activity was recovered in the flowthrough fractions (fraction II). Fraction II (414 mg) was further fractionated on an S-sepharose column (2.5 × 20 cm, 5 mg protein per ml resin). The bound proteins were eluted with an 80 ml linear gradient of KCl (0.1–1.0 M) in buffer C. The active fractions eluting around 0.45 M KCl were pooled and dialysed overnight against buffer C containing 0.1 M KCl (fraction III). Proteins present in fraction III (80 mg) were precipitated by the addition of solid $(\text{NH}_4)_2\text{SO}_4$ (0.43 g ml^{–1}), as described above, and collected by centrifugation at 25×10^3 r.p.m. for 45 min using a Sorvall T647.5 rotor. The pellet was dissolved to 4 ml using buffer C and the solution was centrifuged at 25×10^3 r.p.m. for 20 min using a Sorvall T647.5 rotor. Proteins were then loaded onto a gel filtration column (2.6 × 80 cm) using AcA 44 resin (Fisher-Spectrum). The column was equilibrated with buffer C containing 1.0 M KCl and 0.01% Nonidet P-40 and was developed at a flow rate of 20 ml h^{–1}. Every other fraction of the column was dialysed against buffer C containing 0.1 M KCl and an aliquot (4 µl) of the fractions assayed for TFIIB activity as described below. The active fractions, which eluted with an apparent M_r of ~30K were pooled (fraction IV). Fraction IV (12 mg) was dialysed overnight against buffer C containing 1.0 M $(\text{NH}_4)_2\text{SO}_4$ and debris was removed by centrifugation at 25×10^3 r.p.m. for 25 min using a Sorvall T647.5 rotor. The supernatant was applied to a Bio-Gel TSK phenyl-SPW column (7.5 × 75 mm) that was equilibrated with buffer B (0.1 M Hepes–KOH buffer, pH 7.0, 1 mM DTT, 20% (v/v) glycerol) containing 1.0 M $(\text{NH}_4)_2\text{SO}_4$. Proteins were eluted from the column with a decreasing linear gradient of ammonium sulphate (1.0–0 M) in buffer B. TFIIB activity eluted at 0.4 M $(\text{NH}_4)_2\text{SO}_4$ (fraction V). Fraction V (3.6 mg) was dialysed overnight against buffer C containing 0.1 M KCl and centrifuged at 25×10^3 r.p.m. for



25 min using a Sorvall T647.5 rotor. The supernatant was loaded onto a hydroxylapatite column (7.8 × 100 mm) which was equilibrated with a buffer composed of 10 mM KPI, pH 7.6, 0.1 mM EDTA, 1 mM DTT and 20% (v/v) glycerol. The column was washed with the above buffer containing 0.15 M KPI (ref. 32). TFIIB activity was eluted from the column with a wash of 0.4 M KPI using the above buffer (fraction VI). TFIIB in fraction VI (250 µg) was further purified by chromatography on a 1 ml phenyl superose column as described above for the Bio-Gel TSK phenyl-SPW column. This procedure isolated 30 µg pure TFIIB protein as determined by amino-acid analysis.

peptide of 316 amino acids of calculated M_r of 34,832. This value is in close agreement with the apparent M_r of TFIIB estimated by SDS-PAGE and gel filtration^{5,9}. The sequence of the TFIIB-cDNA clone contains all of the TFIIB-derived peptide sequences and an AATAAA polyadenylation signal (Fig. 2).

Recombinant TFIIB protein

To analyse whether the TFIIB-cDNA clone encoded a functional TFIIB protein the cDNA was expressed in bacteria. *Escherichia coli* cells transformed with a T7-expression vector containing the TFIIB-cDNA clone produced an abundant polypeptide of about 33K (Fig. 3a, lane 5). This polypeptide was absent in bacterial cells transformed with the parental T7-expression vector (Fig. 3a, lane 1). The recombinant TFIIB (rTFIIB) protein fractionated on a phosphocellulose column in a fashion similar to that described for the HeLa cell-derived TFIIB, as rTFIIB was found primarily in the 0.5 M KCl wash (Fig. 3a).

HeLa cell-derived TFIIB can associate with promoter sequences by recognizing the DA complex, a putative intermediate during formation of a transcription-competent complex^{4,5}. The ability of rTFIIB to associate with the DA complex was analysed using the gel mobility shift assay. Under these conditions the rTFIIB yielded a DAB complex which was indistinguishable from that formed with HeLa cell-derived TFIIB (Fig. 3b, compare lanes 6–8 with 9). This complex was absent when a protein fraction derived from cells transformed with the parental plas-

mid was used (pNull, lanes 3–5). The DAB complex provides a foundation site for the association of RNA polymerase II, which is dependent on TFIIF (ref. 50). The rTFIIB protein produced a DAB complex that was recognized by the RNA polymerase II/TFIIF complex as it produced a DABPoIF complex which was indistinguishable from that formed with human TFIIB (Fig. 3b, compare lanes 10 and 11). Consistent with the above results demonstrating that the TFIIB protein produced in *E. coli* replaced the human TFIIB protein in complex formation, the recombinant protein could direct basal levels of transcription from the Ad-MLP (Fig. 3c). Extracts derived from cells containing the parental plasmid were unable to direct transcription (Fig. 3c, lanes 3–6).

Mediation of transcriptional activation by rTFIIB

The isolation of cDNA clones encoding the TATA-binding protein resulted in the observation that transcription reactions reconstituted with bacterially produced TFIID could not support activation²². Thus we analysed whether reactions containing all human-derived factors but bacterially produced TFIIB protein could support activation. The results presented in Fig. 4a demonstrate that the adenovirus major late transcription factor (MLTF)²³ could stimulate transcription from the Ad-MLP (pML) in the presence of rTFIIB protein. Stimulation reached similar amounts to those observed with human TFIIB (data not shown). The activation observed from the MLP was dependent on the MLTF recognition site and MLTF protein, as transcription from a plasmid DNA containing a deletion of this site

FIG. 2 Nucleotide sequence of a cDNA encoding TFIIB (single-letter amino acid code).

METHODS. TFIIB (9 µg) destined for proteolytic cleavage was dissolved in 50 µl 8 M urea, 0.4 M NH_4HCO_3 and reduced with 5 µl of 45 mM DTT at 50 °C for 15 min³³. Cysteine residues were alkylated by reaction with 5 µl 100 mM iodoacetic acid at room temperature for 15 min. S-carboxymethylated TFIIB was diluted fourfold without further desalting or transfer to a final buffer concentration of 2 M urea, 0.1 M NH_4HCO_3 . Endoproteinase LysC was added to this solution to maintain a substrate to enzyme ratio of 25:1 (w/w), and the mixture was allowed to incubate at 37 °C for 16 h. The resultant peptide mixture was chromatographed on a Hewlett-Packard 1090 HPLC equipped with a 1,040 diode array detector, using a Vydac 2.1 mm × 150 mm C18 column. Briefly, where buffer A was 0.06% trifluoroacetic acid/ H_2O and buffer B was 0.055% trifluoroacetic acid/acetonitrile, a gradient of 5% B at 0 min, 33% B at 63 min, 60% B at 95 min and 80% B at 105 min with a flow rate of 150 µl min⁻¹ was used. Chromatographic data were acquired at 210 nm, 277 nm, and ultraviolet spectra from 209 to 321 nm for each peak fraction collected. Fractions for N-terminal sequence analysis were applied directly to a polybrene precycled glass fibre filter and placed in the reaction cartridge of an ABI 477A protein sequencer. The samples were subjected to automated Edman degradation using the standard program NORMAL-1, with the following modifications for a faster cycle time (36 min). The reaction cartridge temperature was raised to 53 °C during coupling with a commensurate decrease in the three R2 delivery steps from 400 to 250 s. The resultant phenylthiohydantoin amino-acid fractions were manually identified using an on-line ABI Model 120A HPLC. A pair of degenerative primers was synthesized on the basis of the amino-acid sequence present in the C terminus of peptide labelled a (MMNAFK); (1) dCACATATGATGAA(C/T)GC(A/C/G/T)TT(C/T)AA, (2) dCAAAGCTT(A/G)AA (A/C/G/T)-GC(A/G)TTCATCAT. Two other degenerative primers were designed based on the amino-acid sequence present in peptide labelled c (QKEIGDI for 3, and GVADVTL for 4); (3) dCA(A/G)AA(A/G)GA(A/G)AT(A/C/T)GG(A/C/G/T)-GA(CT)AT, (4) dAT(A/C/G/T)GT(A/C/G/T)AC(A/G)TC(A/C/T)GC(A/C/T)AC(×A/CT)CC. Primers 1 and 4 were used for PCR using a human cDNA library and the separated products on agarose gel (1%) were transferred to a nitrocellulose membrane and probed with ³²P-labelled primer 3 (refs 34, 35). The positive bands were isolated from a polyacrylamide gel (6%) and subcloned into pUC18 (ref. 36). A positive subclone was selected by colony hybridization (pLA24). Based on the nucleotide sequence of pLA24, an oligonucleotide (dTACATGGCTCACACAGGC, labelled 5 in the figure) was synthesized and used for screening using a HeLa cell cDNA library (Stratagene). Four positive clones were selected from 10⁶ plaques. The clone having the largest insert (about 1.7 kilobase pairs) was selected (pIIIB1) and sequenced using the dideoxy method³⁷.

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GGAATTCCTCTCTTTATTGTGAGGGTCTCTCCCTAGGAGCGCTGCCCGCTAACCGGC 60
TTTTGGCCCAATGGGGCATTATCGAAGATTCACAAAAACATAGCGTCATCATCCCC 120
ACCATCATAGCCACCATCACCCCTCTTAACTCTACTTCTACCTACGCGTAATCTACTCC 180
ACCTCAATCACACTACTCCCCATATCTAACACGTAATAAATGACAGTTTAAACATA 240
CAAAACCCACCCATTCTCCCGACACTCTGCGCCCTTACACGCTACTCTACTATCT 300
CCCTTTTATACATAATATCTCTGTTGTCTTGTTCGGCGACCGAGTCGCCGTGAAG 360

ATGTCGCTCACAGCCGTTTGGATGCTCTTCCAAAGTACATCTCCAAACCATCCAGAT 420
M A S T S R L D A L R V T C P N H C D 20

GCGATTTTAGTGAGGACTACAGAGCCGTTGATATGATCTGCTGAATGGCTGGT 480
A I L V E D Y R A G D M I C P E C G L V 40

GTAGGTGACCGGTTATTGATGTGGATCTGAATGGCAATTTTACGCAATGACAAAGCA 540
V G D R V I D V G S E W R T F S N D K A 60

ACAAAGATCCATCTCGAGTTGGAGATTCTCAGAACTCTCTTGAGTGATGGAGTTTG 600
T K D P S R V G D S Q N P L S L D G D L 80

TCTACCATGATTGGCAAGGGCAGGAGCTGCAAGTTTGGCAATTTGGCAATTTCTAAG 660
S T M I G K G T G A A S F D E F G N S K 100

TACCAGAACTGGAGAACATGAGCAGTTTCTGATCGGGCAATGATGAATGATCAAGAA 720
Y Q N R R T M S S D D R A M M N A F K E 120

ATCATCATCGGACAGCAATCAATCTACCTCGAATATGTTGATGAACAAATAAT 780
I T T M A D R I N L P R N I V D R T N N 140

TTATTCAAGCAAGTATATGAACAGAGAGCTGAAGGGAAGAGCAATGATGCTATAGCT 840
L F K Q V Y E Q N L K G R A N D A I A 160

TCTGCTGTCTATATTGCGCTAGACAGAAAGGGGTTCTAGGACATTTAAAGAAATA 900
S A C L D Y I A C R T T Q K R A N T F K E 180

TGTGCGGTATCAGCAATTTCTAAGAAAGAAATTTGGTGGTGTTTAACTATTTTGAAA 960
C A V S R I S K K E I G R C F K L I L K 200

GCGCTAGAAACCAAGTGTGGATTGATTACAAGTGGGAGCTTCATGTCAGGTTCTGTTCC 1020
A L E T S V D L I L T T G D F M S R F C S 220

AACCTTTGTCTTCTTAAACAGTACAGATGGCAGCTACACATATAGCCCGTAAAGCTGTG 1080
N L C L P K Q V Q M A A T H I A R K A V 240

GAATTTGACTTTGTTCTGGGAGGAGCCCATCTCTGTGGCAGCGGAGCATTTTACATG 1140
E L D L V P G R S P I S V A A A A I Y M 260

GCCTCAGGCTCAGCTGAAAGAGGAGCCCAAAAGAAATTTGGAGATTTTGGTGGTGT 1200
A S Q A S A E K R T T Q K R T Q K D I A G V 280

GCTGATTTACAATCAGACAGTCTATAGACTGATCTATCTCGAGCCCGACATCTGTTT 1260
A D V T I R Q S Y R L I Y P R A P D L F 300

CCTCAGACTTCAAATTTGACACCCCGAGGACAAATCACCACAGCTTAAATTGAGGCA 1320
P T D T F K F D T P V D K L P Q L 316

GCTAACGCTCAAAATTTGAATACAAAATTTGCGTGTGTACATAGCCTATACAAAATGC 1380
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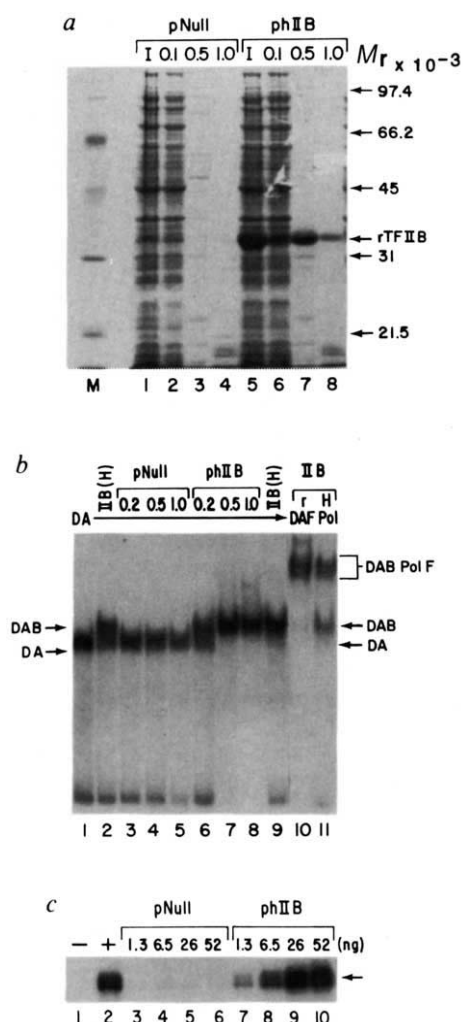



FIG. 3 Expression of recombinant TFIIB in *E. coli* cells. **a**, Coomassie blue staining of a polyacrylamide-SDS gel containing proteins from extracts (or derived thereof) of bacteria transformed with a plasmid allowing expression of the TFIIB-cDNA clone (pIIIB) or the parental plasmid (pNull) (lanes labelled I). The extracts were fractionated on phosphocellulose columns and proteins were eluted with different KCl washes, as indicated at the top of the panel. The phosphocellulose 0.5 M KCl protein fraction was assayed for DNA binding using the mobility shift assay (**b**) and for transcription of the Ad-MLP (**c**). DNA binding and transcription assays were done as described in the legend to Fig. 1.

METHODS. Plasmid pIIIB was generated as follows. N- and C-terminal primers were synthesized such that the PCR product was flanked by *Nhe*I and *Bam*HI sites, respectively (N-terminal, 5'-CGTGGCTAGCACC CGCGTTT-GGATGC-3', C-terminal, 5'-CGGGATCCGACGTTAGCTGCCTC-3', the underlined nucleotides represent the *Nhe*I and *Bam*HI sites). The PCR products were digested with *Nhe*I and *Bam*HI and cloned into the *Nhe*I-*Bam*HI sites present in plasmid pET11a (Novagene). *E. coli* strain BL21 (DE3, Novagene) containing pIIIB or the parental plasmid was cultured in LB media supplemented with ampicillin ($100 \mu\text{g ml}^{-1}$) at 37°C . Cells were induced with isopropylthiogalactoside (0.4 mM) when an OD_{600} of 0.6 was reached. After 2 h, cells were collected by centrifugation at $4,000g$ for 10 min. The pellet was resuspended in 1/20 of the original culture volume using 10 mM Tris-HCl buffer, pH 8.0, 25 mM EDTA and stored at -70°C . The frozen cells were thawed and broken by sonication on ice. Cell debris was removed by centrifugation at $6,000g$ for 15 min and the supernatant was loaded onto two identical phosphocellulose columns (5 mg protein per ml resin) which were equilibrated with buffer C containing 0.1 M KCl. Proteins were eluted from the column as described in Fig. 1.

(ΔpML1) was not stimulated by MLTF. The amounts of transcription from ΔpML1 DNA decreased whereas the amounts of transcription from the wild-type construct increased in an MLTF-dependent fashion. The decreased amounts of transcription from the mutant promoter in the presence of MLTF is probably due to limiting general transcription factors and the fact that MLTF stimulates the formation and stability of transcription-competent complexes²⁴. The stimulation by MLTF with recombinant TFIIB protein was not particular to this activator, as Sp1 could also stimulate transcription with bacterially produced TFIIB (Fig. 4b). The conditions used to analyse Sp1 stimulation were similar to those used to analyse stimulation of transcription with recombinant human TFIID (refs 6, 22). Consistent with the observations that the initiator DNA element can mediate the formation of a TFIID-dependent transcription competent complex^{25,26}, a TATA-less chimaeric promoter containing six Sp1 recognition sites upstream of the

TFIIB is encoded by a single gene

RNA blot analysis of a total RNA fraction or poly(A)-selected RNA, using the TFIIB-cDNA probe, revealed a discrete RNA species of about 1.7 kilobases (kb) which was enriched in the poly(A)-selected RNA (Fig. 5a).

To analyse whether TFIIB was a member of a multifamily of

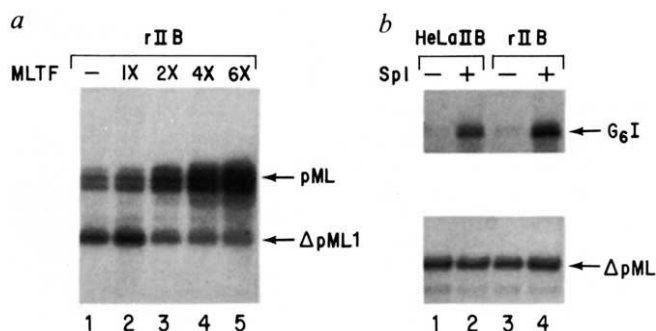


FIG. 4 MLTF and Sp1 proteins can mediate activation of transcription in assays reconstituted with rTFIIB. Transcription assays were done as described in Fig. 1. **a**, Reaction mixtures contained the different general transcription factors, rTFIIB (20 ng), DNA affinity purified MLTF, and two different Ad-MLP DNA templates (100 ng each). Plasmid DNA pML contained the MLTF recognition site and transcription from this template generated an RNA molecule of 390 nucleotides long. Plasmid DNA ΔpML1 contained a deletion of the MLTF recognition site and transcription from this template produced an RNA molecule of 340 nucleotides long. **b**, Transcription reaction mixtures were as described above but the activator was Sp1 (4 ng). The template DNA used in the upper panel was G₆I (ref. 22), whereas the template in the bottom panel was an Ad-MLP construct with a deletion of the upstream element.

genes or was encoded by a single gene, genomic DNA isolated from HeLa cells was digested with each of three restriction endonucleases and blots of separated DNA fragments were probed with the TFIIB-cDNA clone (Fig. 5b). The probe in each case hybridized primarily to a single band, suggesting that there is only one human gene that encodes TFIIB.

Motifs present in TFIIB

Computer search of the Swissprot data base failed to detect any genes with sequence similarity to the TFIIB protein. Previous studies have suggested that TFIIB may be equivalent to BTF3, a factor that seems to be required for transcription of class II genes²⁷. But comparison of the sequence reported for BTF3 (ref. 28), with that encoding TFIIB shows they are different, demonstrating that these two factors are not related.

Analysis of the TFIIB protein sequence, however, revealed a region with similarity to the prokaryotic σ factors and the β -subunit of RNA polymerases present in some RNA viruses (Fig. 6a). The similarity of σ factor mapped to subregion 2.4, which seems to be involved in contacting the -10 region of bacterial promoters²⁹. The relevance of this similarity is unknown. TFIIB binds strongly to single-stranded DNA (ref. 10), but the protein does not recognize any specific sequences on the DNA. Nevertheless, when TFIIB associates with the DA complex, a footprint around the transcription start site on the Ad-MLP is observed^{4,5}. Whether this motif in TFIIB (residues 190–210) is responsible for DA complex association or for the single-stranded DNA binding activity awaits further analysis.

Interestingly, a repeated motif of 76 amino acids (45% similarity and 22% identity) was detected towards the carboxy end of the TFIIB sequence (Fig. 6b). The importance of this repeat for TFIIB function is unknown; however, it is interesting that the sequence of the human and yeast TFIID proteins also revealed a repeated motif³⁰. It is possible that the repeated motif present in TFIIB is functionally similar to that present in the TFIID protein and represents a core element for interaction with the other general transcription factors. The amino terminus may then be involved in contacting activator proteins. The residues 185–202, which map in the first repeat, predict an amphipathic α -helical structure with hydrophobic amino acids on one side of the helix and hydrophilic, mostly basic amino acids, on the other side (Fig. 6c).

The hydropathicity profile of TFIIB shows the N- and C-terminal domains of the protein to be relatively hydrophilic ($pI=8.32$) without any special distribution of acidic or basic amino acids (Fig. 6d, e). TFIIB protein is predominantly hydrophilic between residues 50 to 150 with two small areas of relatively hydrophobic flanking residues. A relatively hydrophobic region is located between residues 200 to 250 (Fig. 6d).

Discussion

In these studies we report the isolation of a human cDNA clone encoding the general transcription factor TFIIB. Expression of the cDNA clone in *E. coli* produced a protein with a M_r of about 33K containing all the functions attributed to TFIIB.

TFIIB, like most of the general transcription factors (with the exception of TFIID), does not recognize any specific sequences in the DNA and seems to be drawn into the transcription cycle by protein-protein interactions. It has been postulated that TFIIB has a structural role during preinitiation complex formation and may be responsible for fixing the initiation site of transcription⁴. Consistent with this putative role is the observation that TFIIB, when in association with the DA complex contacts the DNA at around $+1$. Also, TFIIB structural analyses have detected a short amphipathic α -helix with basic amino acids in one face of the helix and hydrophobic residues in the other. The DAB complex provides the foundation for association of RNA polymerase II/TFIIF complex. It is thus conceivable that the basic amino acids on one side of the α -helix could be contacting DNA in the DAB complex, whereas the hydrophobic

residues on the other side may be interacting either with TFIID or with RNA polymerase II and/or TFIIF. The overall apparent structure of TFIIB, with its 76 amino-acid repeat towards the C-terminal domain and 'tail' at the N-terminus, is similar to the structure reported for TFIID (ref. 6). On the basis of this architectural similarity we suggest that TFIIB is composed of two domains, a repeated domain that participates in basal transcription by contacting DNA and the general transcription factors and a hydrophilic 'tail', which could participate in interaction with activators. In support of this hypothesis is the observation that TFIIB seems to interact with the VP16 activator protein; an interaction that can be displaced by salt²¹. The accuracy of the above speculation awaits mutational analysis of the TFIIB protein.

Initiation of transcription at class II promoters, in comparison with the bacterial promoters, is much more complex. At least seven different factors, in addition to RNA polymerase II, are required for basal transcription. Three mammalian general transcription factors have been cloned (TFIID (refs 6, 7), the small subunit of TFIIF (RAP30)¹⁶ and TFIIB). Interestingly, sequence analysis has revealed each factor to have some similarity with σ factors, a family of prokaryotic factors conferring specificity to the bacterial RNA polymerase. RAP30 has homology with a motif in $\sigma 70$ responsible for interaction with the core enzyme. This homology is not fortuitous, as RAP30 interacts with RNA polymerase II (refs 13, 15). More importantly, this interaction accounts for the specific association of RNA polymerase II with the DAB complex, a putative intermediate during formation of a transcription-competent complex^{4,5}. TFIID was found to contain weak sequence similarity with conserved regions of the bacterial σ -factors, specifically the 2.3 and 2.4 subregions of σ

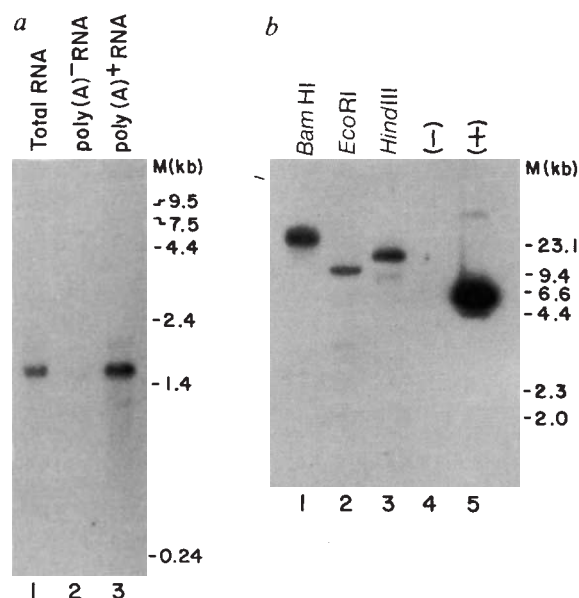


FIG. 5 Northern and southern blot analysis of the human TFIIB gene. *a*, HeLa cell RNA (20 μ g), poly(A)⁻ RNA (20 μ g) and poly(A)⁺ RNA (3 μ g), as indicated, were separated on a denaturing 1.5% agarose gel and transferred to a nitrocellulose filter. After ultraviolet cross-linking the membrane was probed with a nick-translated DNA fragment containing the entire sequence encoding TFIIB (ref. 35). The positions of RNA size markers are indicated on the right side of the panel. *b*, HeLa cell DNA (10 μ g per lane) was digested with the indicated enzymes and separated by electrophoresis on a 0.7% agarose gel. After depurination the DNA was transferred to a nitrocellulose membrane and probed as described in *a* (ref. 38). The filter was washed twice for 20 min at room temperature in 1 $\times$ SSC medium and 0.1% SDS and three times for 20 min at 68 $^{\circ}$ C in 0.2 $\times$ SSC and 0.1% SDS. Lanes labelled (–) and (+) contained *Hind*III-digested λ DNA and pIB1 DNA, respectively. Size markers are indicated on the right side of the panel.

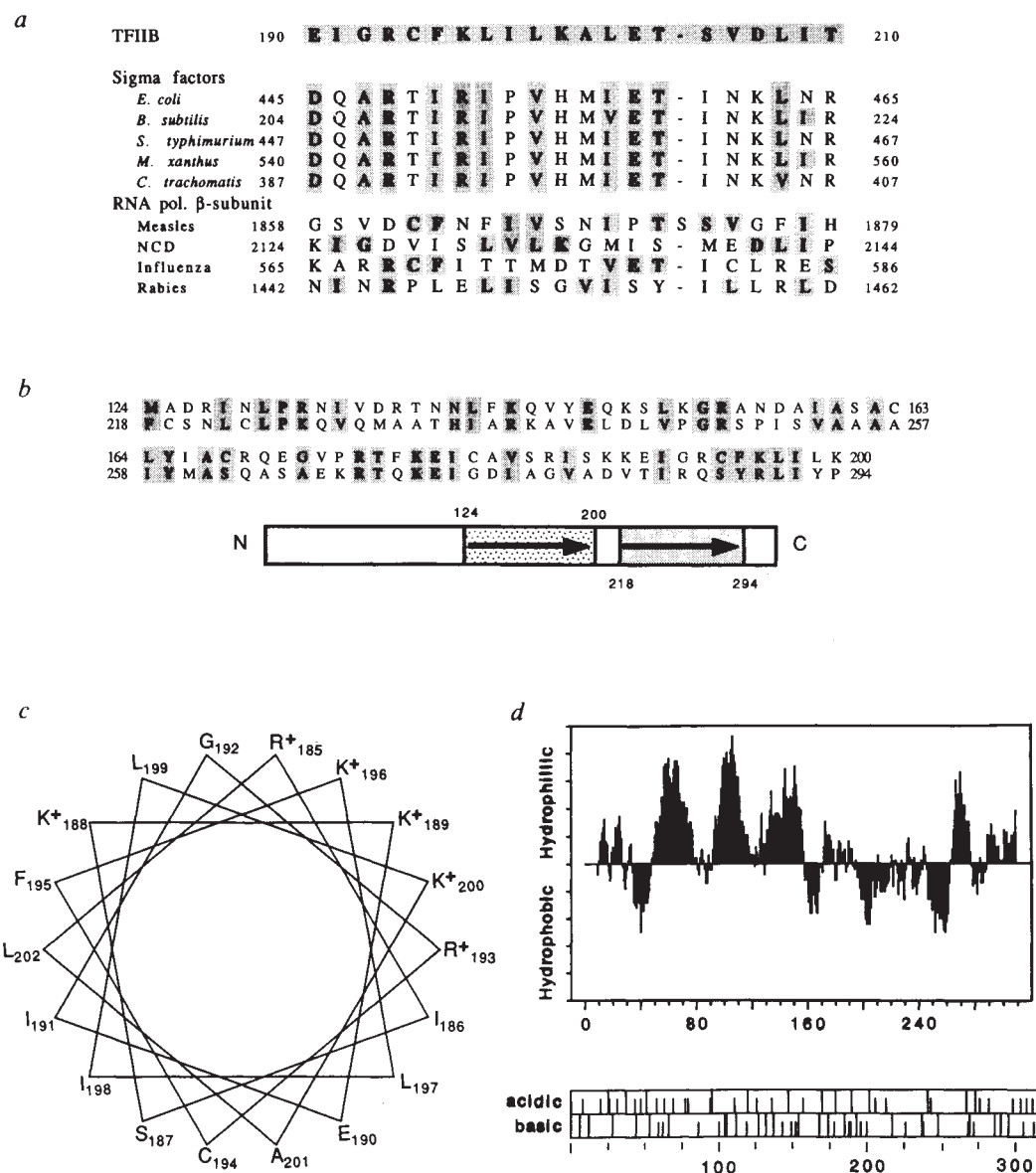


FIG. 6 Structural features of human TFIIB. **a**, Alignment of the amino-acid sequence of TFIIB (residues 190 to 210) with the amino-acid sequences of bacterial (*E. coli*, *Bacillus subtilis*, *Salmonella typhimurium*, *Myxococcus xanthus*, *Chlamydia trachomatis*) σ factors³⁹⁻⁴³ and with the β -subunit of RNA polymerases⁴⁴⁻⁴⁷ present in different RNA viruses, is indicated on the panel. NCD, New castle disease. The amino acids are indicated using the single-letter code. Amino acids identical to those in TFIIB are in bold and conserved amino acids are shaded according to the Gap program of GCG (ref. 47). **b**, Alignment of repetitive sequences in the human TFIIB. The analysis of sequence homology was performed using the Dotplot program. Identical amino acids are denoted with bold characters. Conserved amino acids according to the Bestfit program of GCG are shaded⁴⁸. **c**, Helical wheel depiction of TFIIB residues 185-202. **d**, A linear plot of the hydrophobic and hydrophilic regions of TFIIB using the algorithm of Kyte and Doolittle⁴⁹. The numbers at the bottom denote positions in the TFIIB protein. **e**, Positions of acidic and basic amino acids in TFIIB. Short and long bars denote amino acids D or K and E or R, respectively. Numbers indicate positions in the TFIIB protein.

factors⁸. The similarity between TFIID and the σ subregion 2.4 is striking as this region in σ seems to be involved in contacting the -10 region of bacterial promoters, which bears similarity to the -30 region present in class II promoters and is recognized by TFIID (ref. 29). TFIIB also has a region homologous with subregion 2.4 of σ factors. The relevance of this similarity is unknown; however, it is possible that this region may be important for TFIIB in contacting single-stranded DNA after partial melting of the promoter by the binding of TFIID to the -30 region. It is possible that the various domains of σ specifying different functions are contained on several of the general transcription factors.

Received 7 May; accepted 19 July 1991.

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ACKNOWLEDGEMENTS. We thank N. Stone and L. Zawel for reading of the manuscript, W. Seol for advice on gene screening and microbiological works, S. Eagle for the synthesis of oligonucleotides, R. A. Robinson for technical assistance, R. Tijan for the gift of plasmid G₆ and Sp1 protein. This work was supported by the NIH, the National Science Foundation and the American Cancer Society. D.R. was a recipient of an American Cancer Society Faculty Research Award.

LETTERS TO NATURE

Gamma-ray emission from millisecond pulsars in globular clusters

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RECENT observations indicate that a typical globular cluster may contain hundreds of millisecond pulsars^{1,2}. Because millisecond pulsars have some similarities to the Vela pulsar, which is known to be a source of γ -rays, they could also be gamma emitters of modest power. Here I estimate the γ -ray luminosity of globular clusters, assuming that they indeed harbour large numbers of millisecond pulsars. An upper limit obtained by the Cos-B satellite on the γ -ray emission from 47 Tucanae, which contains at least 10 millisecond pulsars¹, is too large to constrain the model presented here, but the estimated luminosities are high enough to be detectable by the recently launched Gamma Ray Observatory. In the near future, γ -ray observations will thus be able to test this model, and the theory of γ -ray emission by millisecond pulsars.

Young radio pulsars are known to be strong γ -ray sources. The Vela and Crab pulsars are two good examples, and Geminga may be another example^{3–5}. Actually, the Vela, Geminga and Crab are the three strongest 100-MeV γ -ray sources in the sky. Although the rotation period and magnetic field at the surface of the neutron star are very different for a millisecond pulsar and a young canonical pulsar like the Vela pulsar, two parameters related to the emission of γ -rays are similar for the two classes of pulsar. The maximum potential drop (ΔV) across the open field lines of the magnetosphere generated by a spinning neutron star is

$$\Delta V \approx 4\pi^2 p^{-2} B_s R^3 c^{-2} \quad (1)$$

where p , B_s and R are the rotation period, surface magnetic field and the radius of the neutron star, respectively and c is the speed of light. The values of ΔV for PSR1937+21 or the pulsar in globular cluster M28 are within a factor of three of that for Vela. The magnetic fields at the light cylinder are also comparable for these two classes (see Table 1). It is possible that the millisecond pulsars also produce substantial amounts of γ -rays, although no γ -ray emission from any millisecond pulsar has yet been detected.

The detection of γ -rays from millisecond pulsars could help us understand the emission mechanisms of millisecond pulsars and the evolution of millisecond pulsars and low-mass X-ray binaries in globular clusters^{6–8}. It could also be used to evaluate the contribution of millisecond pulsars to the diffuse γ -ray background⁹. Because, unlike radio emission, γ -ray emission from pulsars may only be slightly beamed, γ -ray observation may be useful for statistics of millisecond pulsars in globular clusters.

Recent studies indicate that there may be up to 1,000 mil-

lisecond pulsars in one globular cluster^{1,2}. Thus it is possible that globular clusters are strong γ -ray sources owing to the contribution of many millisecond pulsars. The detection of γ -ray emission from globular clusters could be used to deduce the total number, the distribution of spin period and the magnetic field of these millisecond pulsars. This information will certainly help us understand the formation and evolution of millisecond pulsars in the globular clusters (GCs). Here we use the present theoretical γ -ray emission models of a canonical pulsar like Vela to estimate the γ -ray luminosity of a GC.

The Vela and Crab pulsars lost most of their spin-down energy in the form of electron and positron winds. It is likely that most of the spin-down energy of a millisecond pulsar is emitted in the form of electron and positron winds as well. These electron and positron winds could interact with gases or companion stars to produce γ -rays. We refer to this as secondary γ -ray production. It is difficult to estimate the secondary γ -ray emission because there are many uncertainties in the distribution and density of gases, the number of millisecond pulsars possessing a companion, and the masses of these companions. The energy distribution of secondary γ -rays is also not clear. Grindlay and his collaborators have started to search for the hard X-ray tail of secondary γ -ray emission from globular clusters¹⁰. Here, however, we are more interested in estimating the primary γ -ray emission from millisecond pulsars, which is likely to be dominant. Mechanisms for producing primary γ -ray emission as defined here include¹¹: (1) curvature radiation from an accelerated electron or positron moving along the curved magnetic field lines; (2) inverse Compton scattering of an extreme relativistic electron or positron on a soft photon; and (3) synchrotron radiation by extreme relativistic electrons. For Vela, it is known that $\sim 1\%$ of the total spin-down energy is emitted as primary γ -rays.

A millisecond pulsar is generally believed to be spun up by accretion from a companion in a binary. The progenitors seem to be the so-called low-mass X-ray binaries. The shortest rotation period that a recycled pulsar can attain depends on physical models of the inner part of the accretion disk¹². The standard spin-up model gives $p_{\min} \propto B_s^{6/7}$ if we assume that the accretion is always at the Eddington rate and all the neutron stars have nearly equal masses.

A statistical study of millisecond pulsars in GCs shows that there are too few low-mass X-ray binaries in GCs to account for the large number of millisecond pulsars observed in GCs. Besides the models that have been proposed, there is the possibility that GCs contained several low-mass X-ray binaries in the past. If we assume that all the millisecond pulsars in one globular cluster have roughly the same characteristic age ($\tau = p/2\dot{p}$), then we have $p \propto B_s$. Here we assume that the millisecond pulsars in one globular cluster have $p \propto B_s$, which is not very different from the standard spin-up model ($p \propto B_s^{6/7}$). The typical observed characteristic age of a millisecond pulsar is $\sim 10^8$ yr. Here, we adopt the value for PSR1937+21, 2×10^8 yr, for all the millisecond pulsars in GCs.

The distribution of millisecond pulsars with respect to pulsation period is unknown. So far, 28 millisecond pulsars have

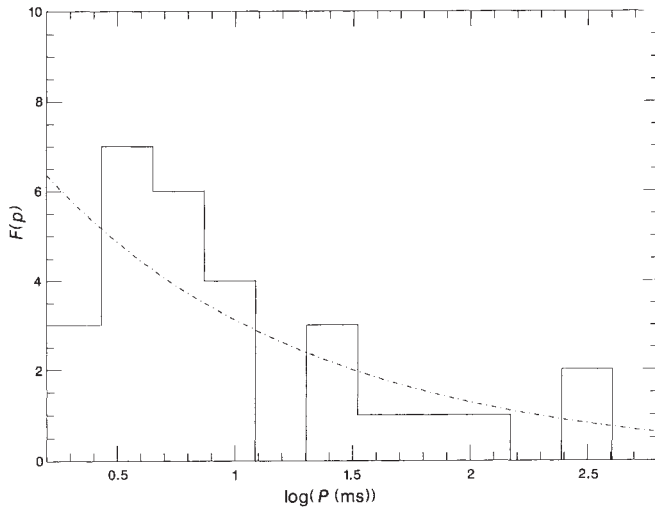


FIG. 1 The observed period distribution of 28 millisecond pulsars in globular clusters fitted by continuous function (dot-dashed line). $F(p)$ is the frequency of occurrence.

been detected in GCs. The distribution of these millisecond pulsars with period (Fig. 1) is certainly not representative, owing to strong selection effects during observation. Nevertheless, this is the only distribution available to us at present. We model the distribution by a simple power-law function $F(p) = (\alpha - 1)p_{\min}^{\alpha-1}p^{-\alpha}$ with $\alpha = 1.4$ for $p > p_{\min} = 1.6$ ms (Fig. 1).

The total spin-down power (P_{total}) of the millisecond pulsars in one GC can be estimated as

$$P_{\text{total}} \sim N \int dp F(p) \frac{32\pi^4 B_s^2 R^6}{3p^4 c^3} \sim 10^{38} n_{500} \quad (2)$$

where $n_{500} = N/500$ is the number of millisecond pulsars in one GC in units of 500. Note that most of the spin-down energy may be emitted as electron and positron winds. Strong electron and positron winds (10^{37} – 10^{38} erg s $^{-1}$) present in globular clusters for long duration (10^8 – 10^9 yr) may have a great effect on the evolution of the stars in the GCs.

If we assume that the efficiency of converting spin-down energy into γ -ray emission is 10^{-3} – 10^{-2} , as in the Crab and Vela pulsars, one expects the γ -ray emission from a GC to be $\sim 10^{35}$ – 10^{36} erg s $^{-1}$.

One also can estimate the γ -ray emission based on detailed theoretical models of γ -ray emission of canonical pulsars. There are some theoretical models of γ -ray emission from the Crab and Vela pulsars, which are in good agreement with observations^{11,13,14}, but there seems to be no theoretical model of γ -ray emission specific to millisecond pulsars. As the potential drop of a millisecond pulsar such as PSR1937+214 is similar to that of the Vela, we assume that millisecond pulsars are Vela-type pulsars. In the 'outer gap' model of Cheng *et al.*¹¹, the ratio, η , of γ -ray luminosity (l_γ) to the pulsar's spin-down energy (L_{sd}) is $\sim 10^{-2}(B_s^{-2}p^5/B_{\text{Vela}}^{-2}p_{\text{Vela}}^5)$ for $\eta < 1$ ($l_\gamma \sim \eta L_{\text{sd}}$). Almost all the spin-down energy will be emitted in γ -rays ($l_\gamma \sim L_{\text{sd}}$) if $\eta > 1$. The spin-down energy is proportional to $B^2 p^{-4}$. The γ -ray

luminosity of a millisecond pulsar is therefore

$$l_\gamma = \begin{cases} 7.8 \times 10^{32} p & p < 14.5 \text{ ms} \\ 2.4 \times 10^{36} p^{-2} & p > 14.5 \text{ ms} \end{cases} \quad (3)$$

where the period p is in milliseconds and l_γ is in erg s $^{-1}$. Thus γ -ray emission from all millisecond pulsars in one GC is

$$L_\gamma = \int dp f(p) l_\gamma = 1.5 \times 10^{36} n_{500} f \quad (4)$$

where L_γ is in erg s $^{-1}$ and f is the beaming factor for γ -ray emission, which is likely to be about unity.

Any GC containing many millisecond pulsars should be a bright γ -ray source. This conclusion is based on our present understanding of the γ -ray emission mechanism from canonical pulsars and it may weakly depend on many assumptions adopted here. Note that the 'outer gap' model predicts the emission of more γ -rays from millisecond pulsars with relatively long period (~ 10 – 15 ms) than from the fastest ones (~ 1.5 ms).

The estimated γ -ray luminosity is just high enough to be detected by earlier γ -ray instruments such as Cos-B¹⁵. As the recently launched Gamma Ray Observatory has much better coverage of energy and greater sensitivity than previous instruments, γ -ray emission from multiple millisecond pulsars in GCs should be detectable, even though most of them may not be detectable individually as radio millisecond pulsars or γ -ray pulsars. Limited angular resolution and the compactness of GCs, may lead to γ -rays from GCs appearing as point sources. We suggest that the GCs should be one of the primary targets for investigation with the Gamma Ray Observatory.

The globular cluster 47 Tuc is particularly interesting because ten millisecond pulsars with period < 6 ms have already been observed there¹. Previous Cos-B observation of the 47 Tuc area provides only an upper limit for the γ -ray flux, of 3×10^{-6} photons cm $^{-2}$ s $^{-1}$ (10^{36} erg s $^{-1}$ for the distance of 4.7 kpc) for photon energy $E > 100$ MeV (J. R. Mattox, personal communication). The upper limit is consistent with our estimation here. Future detailed analysis might reduce this limit to a lower value, but only the Gamma Ray Observatory has the sensitivity to put constraints on the theoretical γ -ray emission mechanism of pulsars and/or challenge the present statistics of millisecond pulsars in globular clusters². □

TABLE 1 Comparison of some millisecond pulsars with Vela and Crab

Name	Period (s)	L_{sd} (erg s $^{-1}$)	ΔV (volts)	B_{lc}^* (Gauss)
Crab	0.033	5×10^{38}	10^{17}	10^6
Vela	0.089	7×10^{36}	5×10^{15}	5×10^4
1937 + 21	0.00155	10^{36}	2×10^{15}	10^6
M28	0.00305	2×10^{36}	3×10^{15}	7×10^5
M4	0.01108	2×10^{34}	3×10^{14}	2×10^4

* Magnetic field at the light cylinder.

Received 4 March; accepted 12 July 1991.

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ACKNOWLEDGEMENTS. I thank J. Arons, D. Bhattacharya, G. Chanmugam, D. M. Eardley, W. Kluźniak, J. Grindlay, J. Halpern, C. Ho, M. Ruderman and J. Shaham for discussions. This work is supported by the NSF.

Early outgassing of Mars supported by differential water solubility of iodine and xenon

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THE martian atmosphere has a high $^{129}\text{Xe}/^{132}\text{Xe}$ ratio compared with any on Earth and most meteorites. The $^{129}\text{Xe}/^{132}\text{Xe}$ ratio in the martian atmosphere is also high relative to the martian mantle¹. In contrast, Earth's upper mantle has a higher $^{129}\text{Xe}/^{132}\text{Xe}$ ratio than its atmosphere². As ^{129}Xe is the daughter product of the extinct nuclide ^{129}I , a means of fractionating iodine from xenon early in martian history appears necessary to account for the $^{129}\text{Xe}/^{132}\text{Xe}$ ratios of its known reservoirs. Crystal/melt partitioning will fractionate iodine from xenon in the right sense, but the fractionation is probably inadequate in magnitude; differences in the silicate melt solubilities of iodine and xenon would cause fractionation in the wrong direction. Here we present a model to account for the martian xenon data which relies on the very different solubilities of the two elements in water to fractionate them after outgassing. Atmospheric xenon is lost by impact erosion during heavy bombardment, followed by release of ^{129}Xe produced from ^{129}I decay in the crust.

The noble gas composition of a planet's atmosphere and surface rocks provides clues to the formation of its atmosphere and the part played by early outgassing of the mantle in this regard. Xenon isotope trends are especially useful. Because of the well-known decay scheme for the production of ^{129}Xe from ^{129}I (half-life $t_{1/2} = 1.6 \times 10^7$ yr), high $^{129}\text{Xe}/^{132}\text{Xe}$ ratios in natural samples are widely regarded as resulting from decay of ^{129}I early in the history of the Solar System^{2,3}.

Table 1 shows the $^{129}\text{Xe}/^{132}\text{Xe}$ ratios for geochemical reservoirs in Earth and Mars. Data for martian basalts are based on measurements of SNC (shergottites, nakhlites, Chassigny) meteorites, which are thought to originate from Mars because of their young ages and because their trapped gases resemble the martian atmosphere, among other factors⁴. A martian origin for SNCs is assumed in this discussion. SNCs have $^{129}\text{Xe}/^{132}\text{Xe}$ ratios falling in the range from 1.0 to 1.5 (excluding pure trapped atmosphere). The actual $^{129}\text{Xe}/^{132}\text{Xe}$ ratio for the mantle source of SNCs is probably at the low end of this range, as martian atmospheric xenon (with its high $^{129}\text{Xe}/^{132}\text{Xe}$ ratio⁵) trapped in the rocks could raise the measured ratio but not lower it. The martian atmospheric ratio is considerably more radiogenic than the source for SNCs. Thus, if SNCs are from Mars, then there is a mantle reservoir on Mars which is less radiogenic than its atmosphere, a situation which is the reverse of the trend for the Earth where the mid-ocean ridge basalt (MORB) source has a higher $^{129}\text{Xe}/^{132}\text{Xe}$ ratio as the Earth's atmosphere². Further-

more, the $^{129}\text{Xe}/^{132}\text{Xe}$ ratio for the martian atmosphere is much higher than for any known Earth reservoir and most Solar System reservoirs. The martian atmosphere is quite thin compared with that of Earth (surface pressure is 6 mbar, compared with 1 bar) and is low in total noble gas abundances⁶. But studies of SNCs⁷ show that the SPB (shergottite parent body, here assumed to be Mars) is two to three times as rich in moderately volatile and volatile elements (excluding noble gases) as the Earth. Either Mars did not outgas its accreted volatiles as completely as Earth, or it has lost most of its outgassed atmosphere, or both.

For the decay of ^{129}I to have an effect on the atmospheric $^{129}\text{Xe}/^{132}\text{Xe}$ ratio, the I/Xe ratio of the atmospheric source needs to be increased above that for the SNC source. Thus, fractionation of iodine from xenon must have occurred while the atmosphere was being formed on Mars. Furthermore, this fractionation must have occurred early in Solar System history, while ^{129}I was extant, as the $^{129}\text{I}/\text{I}_{\text{total}}$ ratio would have dropped by a factor of 100 every 10^8 years after the onset of solar nebula formation. If the atmosphere was formed by mantle outgassing on a timescale of 10^8 yr, then fractionation would have to have increased the I/Xe ratio to 100 times the chondritic value to produce the observed $^{129}\text{Xe}/^{132}\text{Xe}$ ratio in the martian atmosphere (as we show below).

Experimentally determined values for partition coefficients between mineral and melt⁸ for iodine, $D(\text{I})$, are shown in Table 2 with the lowest published mineral/melt partition coefficients^{9,10} for xenon, $D(\text{Xe})$. Comparison of $D(\text{I})$ with $D(\text{Xe})$ for any given mineral/melt pair indicate that iodine is less compatible than xenon during mantle melting.

The value measured experimentally for the partition coefficient of xenon in any given mineral/melt pair varies widely in different experimental runs^{9,10}. This variation makes it difficult to assign specific geochemical significance to the partitioning of the noble gases. The data indicate that xenon is surprisingly compatible, or at least not very incompatible. We assume that the lowest values (most incompatible) for xenon are correct, as it would be difficult for xenon to have degassed from the Earth's mantle if it were compatible in mantle minerals, and degassing of Earth's MORB mantle source clearly has occurred².

Iodine is decidedly incompatible, the crystal concentrations being so low that only upper limits are available in most cases. Although the lowest xenon partition coefficients show xenon to be incompatible, it is not as incompatible as iodine. As a bracketing case, we assume that $D(\text{Xe}) = 0.5$ (the highest value listed in Table 2) and $D(\text{I}) = 0$. At infinitesimal melt fraction, the I/Xe ratio in the melt is infinite. This ratio decreases rapidly, however, with finite melt fractions, such that 0.5% melt is the highest degree of melting that can generate a I/Xe ratio in the melt 100 times that in the solid portion of the mantle. For higher melt fractions, the increase in the I/Xe ratio would be much lower. For instance, a 5% partial melt will lead to only a ten-fold increase in the I/Xe ratio in the melt fraction. This fractionation is in the right direction, but it is too low unless Mars experienced essentially no partial melting during accretion. Uranium-lead fractionation at 4.6 Gyr (ref. 11) indicates that core formation was contemporaneous with accretion, and the segregation of metals and silicates seems to require high degrees of partial melting of the silicate mantle¹².

We next consider fractionation of iodine from xenon during magma outgassing. Table 2 gives a preliminary value for the

TABLE 1 Ratios of ^{129}Xe to ^{132}Xe in Earth and Mars reservoirs

	Mars ^{1,18}	Earth ²
Atmosphere	2.4	0.985
Basalts	1.0–1.5	0.988 OIB* 1.00–1.14 MORB*

* OIB refers to ocean island basalts, MORB refers to mid-ocean ridge basalts.

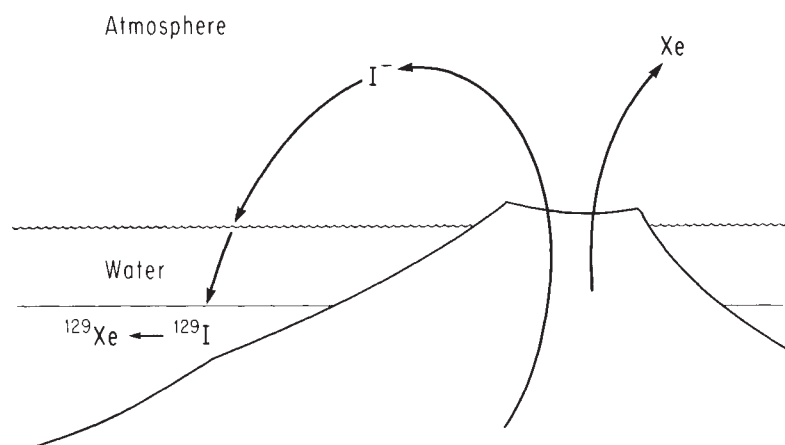


FIG. 1 Mars outgassing model. Accretion ceases at 4.45 Gyr and liquid H_2O becomes stable; iodine and xenon are both outgassed from SNC source; iodine dissolves in the ocean and is incorporated hydrothermally into the crust; xenon remains in the atmosphere; early heavy bombardment erodes the atmosphere, leaving most liquid water standing, so that iodine dissolved in the water is retained, but the xenon in the atmosphere is removed; ^{129}I in the crust decays to ^{129}Xe ; ^{129}Xe (and ^{40}Ar) is released over geologic time.

silicate melt solubility of iodine, from experiments that we have in progress, together with the solubility for xenon. The silicate melt solubility of iodine is several orders of magnitude higher than that for xenon. The I/Xe ratio in the outgassed portion of the mantle will therefore increase. In the case of a substantial fraction of the SNC mantle source being outgassed, the fractionation in the melt becomes significant and the I/Xe ratio for the mantle would be greater than the atmosphere. Although the solubility of iodine is greater than that of xenon, it is low in an absolute sense. Because of the low abundance of iodine, its partial pressure in a vapour phase in equilibrium with a melt will be low, and thus its absolute silicate solubility will be low. Both xenon and iodine would be outgassed from the melt, but the I/Xe ratio in the atmosphere will not increase because of the difference in solubilities.

The inability of iodine and xenon to fractionate during magmatic processes indicates that a way of fractionating the two elements after outgassing needs to be considered. One possibility is differential solubility in water. Table 2 shows a range of values for the solubility of iodine and xenon in water for the range of temperatures under which water is stable and for likely partial pressures of xenon. Both solubilities are expressed as saturation concentrations for ease of comparison. The solubility of iodine, 27 g l^{-1} , is very high, indicating that any outgassed iodine would eventually dissolve in a standing body of water. The solubility for xenon is at least 10^{10} times smaller, indicating that xenon would not go into solution. Thus, differences in water solubilities provide a mechanism for a nearly complete separation of iodine from xenon. The existence of runoff channels in ancient cratered terrain on Mars suggests that liquid water was stable at the martian surface early in its history¹³.

We propose a model for Mars (Fig. 1) which starts with condensation of the solar nebula and accretion of the planets from planetesimals commencing at 4.55 Gyr and proceeding to

4.45 Gyr. During the process of accretion, we assume that Mars developed a steam atmosphere¹⁴, although this is not essential to the model. As late as 4.45 Gyr (this is a conservative estimate), accretion ceases, the steam atmosphere condenses, and liquid water is stable at the martian surface. Outgassing of the SNC mantle source will lead to substantial outgassing of both iodine and xenon, because of their low solubilities in silicate melts. Iodine is highly soluble in water and would go into solution. Its short residence time of 4×10^5 years for present terrestrial oceans¹⁵ suggests that it would be rapidly incorporated into the crust. Xenon, with an extremely low solubility in water, would be partitioned into the atmosphere. Calculations¹⁶ show that impact erosion of the atmosphere from early heavy bombardment is considerable for Mars but not for Earth. This impact erosion would diminish the martian atmosphere to nearly its present level by 4.0 Gyr ago. As the atmosphere is probably not substantially heated by this process, water not directly involved in impacts will remain standing. Iodine-129 residing in the crust will decay to ^{129}Xe , and will be released over geologic time into the now diminished atmosphere, thereby increasing the atmospheric $^{129}\text{Xe}/^{132}\text{Xe}$ ratio.

In our model for Mars outgassing, events are described as a series of discrete steps for simplicity. In the real Solar System, accretion would not have ended before outgassing begins. On the contrary, considerable outgassing must occur during accretion, but this is irrelevant to the outcome of the model, as iodine outgassed into a steam atmosphere will go into aqueous solution when the steam condenses.

The timing of the events described above is crucial, however, given the short half-life of ^{129}I . Is the interval of 10^8 years after accretion has started already too long? At the start of Solar System formation³, $^{129}\text{I}/^{132}\text{I}_{\text{total}} = 10^{-4}$. The ratio of $^{129}\text{I}/^{132}\text{I}_{\text{total}}$ after 10^8 years would be $\sim 1.3 \times 10^{-6}$. Although this figure is low, there is enough ^{129}I extant to account for the observed excess ^{129}Xe in the atmosphere because of the high I/Xe ratio in the crust in our model. The I/Xe ratio required to achieve the present day $^{129}\text{Xe}/^{132}\text{Xe}$ ratio in the atmosphere is:

$$(I/Xe)_r = (^{129}\text{I}/^{132}\text{Xe}) \times (^{132}\text{Xe}/Xe_{\text{total}}) / (^{129}\text{I}/I_{\text{total}})$$

where $^{129}\text{I}/^{132}\text{Xe}$ is determined from the observed excess $^{129}\text{Xe}/^{132}\text{Xe}$ ratio relative to the SNC mantle source. The $^{129}\text{I}/I_{\text{total}}$ ratio depends on the timing of outgassing, assumed to be instantaneous for the purpose of these calculations. To account for the $^{129}\text{Xe}/^{132}\text{Xe}$ ratio of the martian atmosphere with complete outgassing occurring by 4.45 Gyr the I/Xe ratio must be 3.1×10^5 . From analyses of SNCs⁷, we estimate that the present I/Xe ratio for Mars is 2.4×10^5 . Assuming that 97% of the atmosphere was removed at 4.45 Gyr¹⁶ before emission of ^{129}Xe from the crust, the outgassed I/Xe ratio would be 8.9×10^3 , or roughly four times the chondritic ratio¹⁷. As discussed above, partitioning between crystal and melt can reasonably generate

TABLE 2 Mineral/melt partition coefficients for iodine and xenon

Mineral/melt partition coefficients*	Iodine		Xenon
	1 bar, 1,300 °C	15 kbar, 1,475 °C	1 Bar, 1,300 °C
Forsterite	≤ 0.04	—	0.14
Enstatite	—	0.016 ± 0.009	—
Diopside	≤ 0.003	≤ 0.02	0.5
Anorthite	≤ 0.02	—	0.27
Silicate melt solubility†	0.2		10^{-5}
Water solubility‡	≥ 27		$\leq 5 \times 10^{-10}$

* I, from ref. 8; Xe, lowest determined values from refs 9, 10.

† I, measured in this work; Xe, from ref. 10. Units are $\text{cm}^3 (\text{STP}) \text{g}^{-1} \text{atm}^{-1}$.

‡ From ref. 20; units are g l^{-1} .

an increase of less than a factor of 10 in I/Xe ratio in a melt. This model can therefore produce the observed present-day I/Xe ratio of Mars from chondritic starting material.

Our model agrees with an earlier assertion⁷ that iodine will have been incorporated into martian crustal rocks formed early in the planet's history, whereas xenon was outgassed and lost. The sequestering of ¹²⁹I-derived ¹²⁹Xe into the martian crust in our model is also consistent with an earlier suggestion¹⁸ that the easiest way to produce SPB xenon is to fractionate xenon from average carbonaceous chondrites and then add ¹²⁹Xe. The

fractionation of this xenon could occur during late accretion, perhaps by hydrodynamic escape¹⁹, or during or immediately after late, heavy bombardment by some other mechanism that also depends on mass. The addition of ¹²⁹Xe would occur by slow release from the crust over geologic time. Outgassing of martian surface rocks following the loss of a substantial portion of the martian atmosphere is consistent with enrichment of ⁴⁰Ar in the martian atmosphere⁵ (⁴⁰Ar/³⁶Ar = 3,000 for Mars, 295 for Earth), which resulted from the release of ⁴⁰Ar produced by decay of ⁴⁰K over geologic time. □

Received 1 July; accepted 2 July 1991.

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ACKNOWLEDGEMENTS. We thank J. Holloway for providing access to a piston-cylinder apparatus at Arizona State University, P. Rogers and T. Benjamin for assistance with the PIXE at Los Alamos National Laboratory, C. Capobianco for help with experimental procedure, M. Ozima, M. Caffee, R. Pepin, Y. Zhang and L. Broadhurst for helpful discussions, and NASA for financial support.

Paradoxical behaviour of mechanical and electrical networks

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WE describe here a network of strings and springs in which cutting a string that supports a weight results in a rise of the weight at equilibrium. In an analogous electronic circuit of passive two-terminal devices (resistors and Zener diodes), adding a current-carrying path increases the voltage drop across the circuit. These systems are mechanical and electrical analogues of a paradox of congested traffic flow^{1,2}. Along with similar hydraulic and thermal analogues, they show how non-intuitive equilibrium behaviour can arise in physical networks made up of classical components.

In the network of strings and springs shown in Fig. 1a, one end of a spring is attached to a fixed support, and the other end to a string of length $L = \frac{3}{8}$ m. One end of another identical spring is attached to the free end of the string, and a weight is attached to the free end of the second spring. A back-up string joins the support to the upper end of the second spring, and an identical back-up string joins the lower end of the first spring to the weight. These back-up strings are long enough to be limp when the linking string of length $L = \frac{3}{8}$ m is intact.

Assume the strings are massless and perfectly inelastic, and that the springs are massless, ideally elastic and have zero unstretched length. Assume each spring has a spring constant equal to k so that its extension x is related to the applied force F by $F = kx$. For simplicity, we will take $k = 1$. If the weight exerts a force of $\frac{1}{2}$ N, the extension of each spring will be $\frac{1}{2}$ m. Therefore the distance from the support to the lower end of the linking strings is $\frac{1}{2} + \frac{3}{8} = \frac{7}{8}$ m. The distance from the upper end of the linking string to the weight is the same. The length of the two safety strings is chosen as 1 m so that they hang limply. The distance X from the support to the weight is $\frac{1}{2} + \frac{3}{8} + \frac{1}{2} = 1\frac{3}{8}$ m.

Intuition (ours and that of other people we have asked) suggests that if the linking string were cut, the weight would drop and the distance X would increase. In fact, the opposite is true. If the linking string is cut, the network becomes as shown in Fig. 1b. The safety string attached to the support and the lower spring attached to that string now bear half the weight. The upper spring and the safety string attached to the weight bear the other half of the weight. Therefore the extension of each spring is $\frac{1}{2} \times \frac{1}{2} = \frac{1}{4}$ m, and the distance X from the support to the weight is now $1 + \frac{1}{4} = 1\frac{1}{4}$ m, less than before. At equilibrium, the weight is higher than before. Directionality is irrelevant. The phenomenon would be the same if the network were hung upside down. (When the strings and springs are massless, even the initial transient motion of the weight is upward just after the string is cut.) Of course, at equilibrium, the whole network has less potential energy after the string is cut than before.

Suppose the length of the cross-linking string varies smoothly from 0 to 1 m, while all other elements of the network remain as in Fig. 1a. Figure 2 shows the equilibrium distance from the support to the weight as a function of the length L of the cross-linking string. For any L between 0.25 m and 0.75 m, the weight would be higher (the distance X from the support to the weight would be smaller) if the cross-link were cut.

An electrical analogue of the paradoxical mechanical network may be constructed by associating current (I) with force and voltage (V) with displacement. A spring with zero rest length (which obeys $F = kx$) thus becomes a resistor ($I = V/R$), with resistance R analogous to the inverse of the spring constant, $1/k$. A string (of constant length x , for any $F > 0$) becomes a Zener diode ($V = V_Z$, for any $I > 0$), with its characteristic Zener voltage V_Z analogous to the string's length. These ideal circuit elements can be realized fairly accurately in practice³.

Figure 3a shows the resulting electrical analogue of Fig. 1b, drawn as a Wheatstone bridge. To match the elastic springs and inelastic strings of Fig. 1b, we will consider 1-Ω resistors and 1-V Zener diodes in the outer legs (unrealistic values in practice, but convenient for illustration). A current of $I = \frac{1}{2}$ A flowing into the top terminal divides equally between the two paths, producing a voltage drop of $V = \frac{1}{2}IR_4 + V_{Z1} = \frac{1}{2}IR_2 + V_{Z5} = \frac{1}{4} + 1 = 1\frac{1}{4}$ V. Providing an additional conducting path across the bridge in the form of a $\frac{3}{8}$ -V Zener diode (analogous to the cross-linking string of length $L = \frac{3}{8}$ m) produces the circuit of Fig. 3b, in which all the current flows through R_2 , Z_3 and R_4 , and the voltage drop has increased to $V = IR_2 + V_{Z3} + IR_4 = \frac{1}{2} + \frac{3}{8} + \frac{1}{2} = 1\frac{3}{8}$ volts. No current flows through the 1-V Zener diodes, because the

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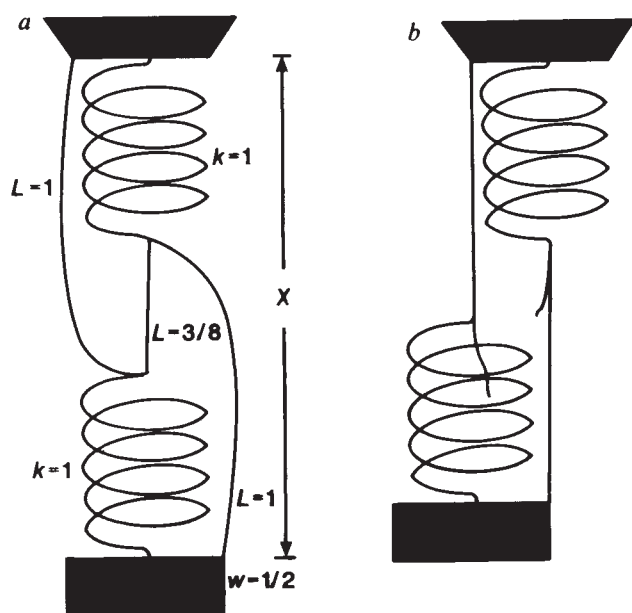


FIG. 1 Mechanical network. Springs have zero unstretched length and spring constant $k=1$. Strings are inelastic. The string that links the two springs has length $\frac{3}{8}$ m. Both safety strings have length 1 m. The weight exerts a force of $\frac{1}{2}$ N. *a*, In the initial network, both safety strings are limp, and the distance X from support to weight is $1\frac{3}{8}$ m. *b*, After the linking string is cut, the weight is higher at equilibrium; the new distance from support to weight is $1\frac{1}{4}$ m.

voltage drop across each is only $\frac{7}{8}$ V. Adding a new current-carrying path, while leaving all previous paths in place, thus decreases the conductivity of the overall two-terminal network.

As in the mechanical network, directionality is irrelevant. Although unidirectional Zener diodes were used in this example, they could be replaced with bidirectional Zener diodes³. The current paths are set by the sign of the forcing function (the applied current) and the circuit topology, and would be identical to those of Fig. 3. In the circuit with bidirectional diodes, the applied current could be of either sign, with similar results (analogous to an interchange of weight and support in the mechanical model).

For the topology of the Wheatstone bridge, it is easy to see under what conditions the paradox occurs. In Fig. 3*b*, let both

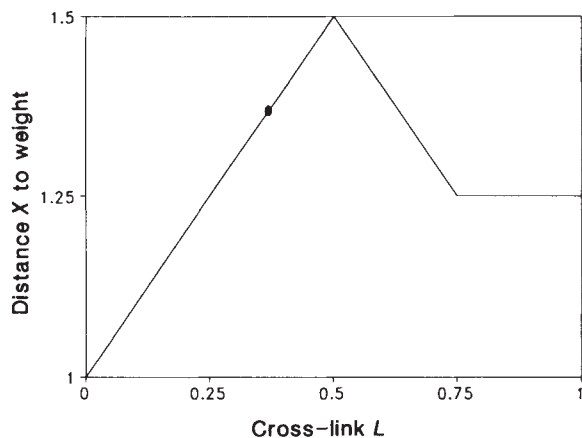


FIG. 2 Distance X from the support to the weight, as a function of the length L of the cross-linking string. For any L between $\frac{1}{4}$ and $\frac{3}{4}$ m, the distance from the support to the weight will be smaller if the cross-link is cut. The dot shows the position of the network in Fig. 1*a*.

the current I (A) and the voltage V_{Z3} (V) of the bridging Zener Z_3 be variable positive numbers. Then a paradoxical change in voltage drop occurs if $1 - 3I/2 < V_{Z3} < 1 - I/2$. The right-hand inequality guarantees that current flows through Z_3 . The left-hand inequality guarantees that the conductivity is reduced by adding the additional path. As $V_{Z3} \geq 0$, I must be less than 2 A. The maximum ratio of the voltage drop with the cross-bridge to the voltage drop without the cross-bridge (or distance X , for the mechanical analogue) is $\frac{4}{3}$, and occurs when $I = 1$ and $V_{Z3} = 0$ (the voltage drop is 2 V with the bridge, $1\frac{1}{2}$ V without it).

An arbitrary two-terminal electrical network with a fixed current source in which all branches contain only strictly linear resistors (those for which $V = IR$) cannot produce this paradoxical behaviour⁴⁻⁶. Consequently neither can a mechanical network in which all elements are massless, ideally elastic springs with zero unstretched length. These general results add force to the surprise produced by the examples in Figs 1 and 3.

Additional paths may cause reduced flow in other electrical circuits in ways that are not counter-intuitive. The circuit in Figure 4, for example, consists of a bipolar transistor and one resistor. When the dashed path is open, or not connected, the circuit conducts heavily for any applied voltage greater than ~ 1 V (or, equivalently, any applied current results in a voltage drop of ~ 1 V). If the dashed path is connected (a conducting path is added), the transistor no longer conducts, and the circuit behaves like a single resistor, R ; the conduction across the circuit is thus greatly reduced. This behaviour is hardly paradoxical, owing to the presence of an active three-terminal device (the transistor). By contrast, the circuits of Fig. 3 use only passive two-terminal devices, and there the increase in voltage drop with an additional current path seems genuinely counter-intuitive.

The networks in Figs 1 and 3 are analogous to a hydraulic

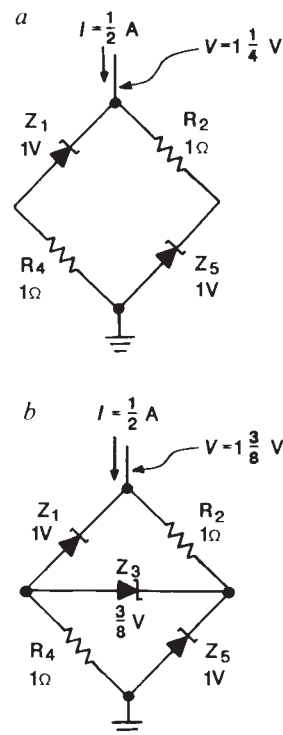


FIG. 3 Electrical network of ideal components. *a*, Initially, current flows symmetrically through left and right branches, and the voltage drop from source to ground is $1\frac{1}{4}$ V. *b*, When a $\frac{3}{8}$ -V Zener diode is introduced across the network, the current through the 1-V Zener diodes drops to zero and all current flows through the 1- Ω resistors and the $\frac{3}{8}$ -V Zener diode, producing a larger voltage drop from source to ground of $1\frac{3}{8}$ V.

network carrying an incompressible fluid in which the inelastic strings or Zener diodes are replaced by constant-pressure-difference valves (sometimes called pressure-relief valves or safety valves) and the elastic springs or linear resistors are represented by tubing of appropriate length with incompressible viscous (Poiseuille) flow; fluid flow is then analogous to weight or electrical current, and pressure difference is analogous to extension or voltage drop⁷. In an analogous thermal network, heat flow corresponds to weight or current, and temperature difference corresponds to extension or voltage drop⁸.

These physical paradoxes are closely connected to a paradox in traffic flow discovered by Braess¹. In an idealized traffic network, each individual seeks a minimal-cost (or shortest) path from a point of entry to a point of exit. In an uncongested network, the choices of paths through the network made by different individuals do not affect one another. Adding uncongested routes to an uncongested network can only lower, or at worst not change, the time individuals require to travel through the network from a source to a destination. A congested network differs from an uncongested one in that, for at least one arc of a congested network, the cost (per person or vehicle) of travel along that arc strictly increases with increasing traffic flow. Braess^{1,2} discovered a congested transportation network such that, if a link is added and all individuals seek their best possible route, at the new equilibrium the cost of travel for all individuals is higher than before.

Braess's paradox (additional capacity leading to more costly travel for all) occurs both in general transportation networks^{9,10} and in a queuing network¹¹. Thus Braess's paradox is not a peculiarity of the mathematical formalism Braess used to describe a transportation network, but appears to be a more general property of some congested flows. Transportation and queuing networks are special examples of non-cooperative games, for which a general analogue of Braess's paradox holds¹²: in the language of game theory, Nash equilibria of non-cooperative games are generically Pareto-inefficient.

Braess's original model translates to a mechanical network similar to Fig. 1, with each inelastic string replaced by an inelastic string in series with a spring; or, analogously, to an electrical circuit similar to Fig. 3, with each Zener diode replaced by a Zener diode in series with a resistor. The translations among transportation networks, mechanical networks, electric circuits and hydraulic networks are exact because there is conservation at every node (traffic in equals traffic out, mechanical force upward equals mechanical force downward at equilibrium, and so on) and because different paths from one node to another, if used, must have equal cost (travel time, stretch, voltage drop or pressure drop). Analogues of Braess's paradox should also exist in continuous representations of systems that obey Kirchhoff's laws.

These mechanical and electrical analogues of Braess's paradox illustrate the possibility of counter-intuitive equilibrium

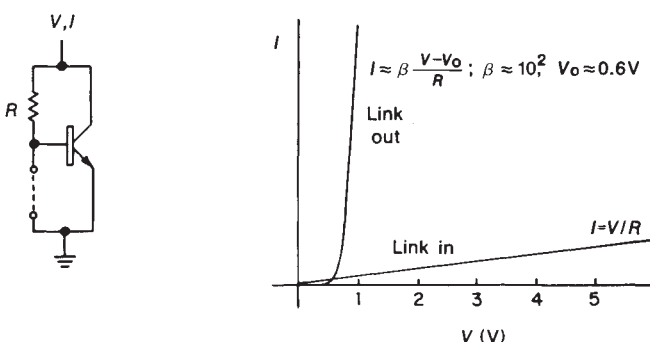


FIG. 4 A less surprising two-terminal electrical network in which an added current path (dotted) causes less current to flow from source to ground. The transistor acts as a switch.

behavior in physical networks when the load imposed on an arc affects that arc's behaviour (stretch or voltage drop, in these examples). The task remains of specifying the general conditions under which such paradoxes can occur, for general network topologies and broad classes of components, possibly including nonlinear ones. The examples presented here suggest caution in assuming that physical networks will behave as normally expected when paths or components are added. □

Received 19 April; accepted 30 May 1991.

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ACKNOWLEDGEMENTS. We thank D. Braess, R. J. Duffin, F. Dyson, R. L. Garwin, S. Glashow, G. L. Miller, R. Muller, G. F. Oster, W. Press, F. Seitz and R. Steinberg for encouragement and helpful suggestions. J.E.C. acknowledges the support of an NSF grant and the hospitality of Mr and Mrs W. T. Golden. P.H. acknowledges the support of the Planetary Society.

Preparation and structure of the alkali-metal fulleride A_xC_{60}

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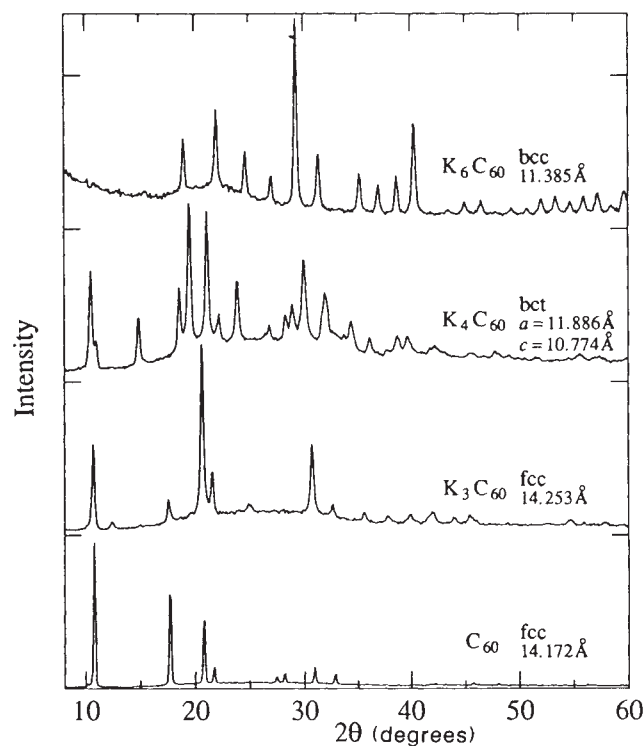
SUPERCONDUCTING K_3C_{60} (ref. 1) has been shown² to have an intercalated face-centred cubic (f.c.c.) structure, and other A_3C_{60} compounds (where A is K, Rb or mixtures of K, Rb and/or Cs) form an isostructural series with superconducting transition temperatures up to 31.3 K (ref. 3). Recently we reported⁴ ^{13}C NMR studies of K_xC_{60} for $x < 3$, and concluded that the system contained two phases ($x = 0$ and $x = 3$) for $0 < x < 3$. Here we report an investigation of A_xC_{60} in the range $3 < x < 6$ and find evidence for an additional phase at $x = 4$. In contrast to f.c.c. A_3C_{60} (refs 2, 3) and body-centred cubic A_6C_{60} (ref. 5), A_4C_{60} (where A is K, Rb, Cs) has a body-centred tetragonal structure. We show that all of the experimentally observed phases of A_xC_{60} can be predicted solely on the basis of electrostatic considerations. We have found no evidence for superconductivity in A_4C_{60} .

Rb_4C_{60} was prepared by reaction of stoichiometric quantities at 225 °C for three days followed by 400 °C for one week in sealed, evacuated Pyrex tubes. K_4C_{60} was prepared by thermal de-intercalation of K_6C_{60} by heating in an evacuated Pyrex ampoule for one week in a thermal gradient with the K_6C_{60} at 550 °C and the cold end at room temperature. After de-intercalation, X-ray diffraction showed the material to be single-phase body-centred tetragonal (b.c.t.). For Cs_xC_{60} , the b.c.t. phase has been seen thus far only in two-phase samples prepared by reaction of material with nominal composition Cs_3C_{60} .

Representative Cu $K\alpha$ X-ray powder patterns of K_xC_{60} in sealed glass capillaries are shown in Fig. 1 for $x = 0, 3, 4$ and 6. C_{60} and K_3C_{60} can be indexed as f.c.c. with the lattice parameters shown^{2,6}, whereas K_6C_{60} is body-centred cubic (b.c.c.)⁵. A_4C_{60} is neither b.c.c. nor f.c.c., but instead can be indexed with a b.c.t. unit cell with lattice parameters of $a = 11.886(7)$ and $c = 10.774(6)$ for K_4C_{60} , and $a = 11.962(2)$ and $c = 11.022(2)$ for

TABLE 1 Observed and calculated parameters for A_4C_{60} (tetragonal $I4/mmm$)

Rb_4C_{60}						K_4C_{60}		
<i>h</i>	<i>k</i>	<i>l</i>	$2\theta_{obs}$	I_{obs}	I_{calc}	$2\theta_{obs}$	I_{obs}	I_{calc}
1	1	0	10.437	160	193	10.502	592	629
1	0	1	10.873	26	13	10.982	169	109
2	0	0	14.785	125	153	14.884	278	343
0	0	2	16.079	149	126	16.43*	0	1
1	2	1	18.42*	0	1	18.627	394	490
1	1	2	19.216	654	767	19.526	1,000	1,000
2	2	0	20.997	1,000	1,000	21.142	827	784
2	0	2	21.865	109	93	22.175	124	155
1	3	0	23.50*	0	0	23.67*	0	8
3	0	1	23.718	631	693	23.926	394	386
1	0	3	25.357	66	91	25.89*	0	13
2	2	2	26.574	310	342	26.844	64	72
2	3	1	28.125	132	202	28.326	144	260
3	1	2	28.688	204	227	28.937	160	298
2	1	3	29.483	592	710	30.024	623	854
4	0	0	29.868	252	302	30.07		
3	3	0	31.730	116	153	31.798	189	133
4	1	1	31.90*	0	5	32.095	263	80
0	0	4	32.482	104	48	33.26*	0	92
3	0	3	33.168	104	61	33.786	40	21
4	2	0	33.50*	0	12	33.718		
4	0	2	34.107	264	355	34.451	183	226
1	1	4	34.22*	0	2	34.94*	0	10
3	3	2	35.777	155	131	36.157	101	75
4	3	1	38.488	237	166	38.799	187	89
5	0	1						
2	2	4	39.023	212	177	39.776	176	98

* Calculated 2θ .FIG. 1 X-ray powder patterns of K_xC_{60} for $x=0, 3, 4$ and 6 .

Rb_4C_{60} . Although we did not prepare Cs_4C_{60} directly, a two-phase sample of nominal composition Cs_3C_{60} contained a b.c.t. cell with lattice parameters of $a = 12.057(18)$ and $c = 11.443(18)$. For $A = K$ and Rb , the b.c.t. phase is seen in equilibrium with f.c.c. for samples with nominal compositions of A_xC_{60} with $3 < x < 4$, but is seen together with a b.c.c. second phase for samples with $4 < x < 6$. This indicates that the $x = 4$ composition is a line phase with little variation in the stoichiometry, as are $x = 0, 3$ and 6 .

The powder patterns of A_4C_{60} (where A is K, Rb, Cs) can be indexed as b.c.t., suggesting a possible space group of $I4/mmm$. We note that if the C_{60} molecules are ordered, the symmetry

can be no higher than orthorhombic (for example, $Immm$) with an accidental degeneracy of a and b . This follows from the lack of a fourfold axis in the C_{60} molecule, which prohibits it from occupying the origin of a b.c.t. cell. For A_4C_{60} to be b.c.t., the C_{60} must be orientationally disordered or rotating rapidly. We have therefore modelled A_4C_{60} for $A = K, Rb$ using $I4/mmm$ with uniform shells of electron density at $(0, 0, 0)$ and alkali-metal atoms at $(0, \frac{1}{2}, z)$. A refinement of z gives a best fit at $z = 0.28$, roughly the same fractional coordinate found⁵ for the alkali-metal positions in A_6C_{60} . The radius of the C_{60} sphere was set to $3.55 \text{ \AA}$, a value previously determined^{2,6} and varied manually. Changes in the sphere radius by $\pm 0.05 \text{ \AA}$ degraded

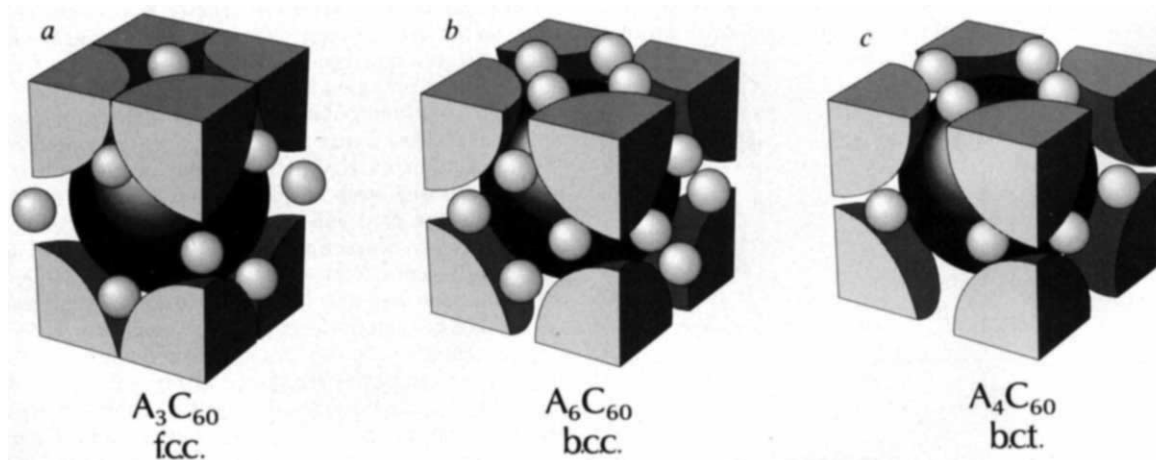
FIG. 2 Structures of A_3C_{60} , A_4C_{60} and A_6C_{60} (schematic). The f.c.c. cell has been rotated by 45° and depicted as an equivalent b.c.t. cell with $a_{bct} = a_{fcc}/\sqrt{2}$.

TABLE 2 Electrostatic calculations for Rb_xC_{60}

x	Phase	Site symmetry	Lattice (Å)	$\text{C}_{60}\text{--C}_{60}$ (Å)	Madlung energy (eV)	Energy of C_{60}^{x-} (eV)	Total (eV)
1	f.c.c.	O	14.436	10.208	-3.49	-2.60	-6.09
2	f.c.c.	T	14.436	10.208	-5.80	-1.57	-7.37
3*	f.c.c.	O and T	14.436	10.208	-7.36	-0.23	-7.59
3	b.c.c.	O	11.787	10.208	-7.23	-0.23	-7.46
3	A15†	T	11.787	10.208	-7.16	-0.23	-7.39
4	b.c.c.	T	11.657	10.095	-8.52	1.18	-7.34
4†	b.c.t.	T‡	11.962 (<i>a</i>) 11.022 (<i>c</i>)	10.095	-8.70	1.18	-7.52
6†	b.c.c.	T	11.548	10.001	-11.74	4.24	-7.50

* Experimentally observed.

† Cubic $Pm\bar{3}n$.

‡ Distorted site.

the quality of the fit. Table 1 summarizes the observed and calculated intensities for K_4C_{60} and Rb_4C_{60} using these parameters.

Figure 2 shows the close relationship between the f.c.c., b.c.c. and b.c.t. sphere packing and the sites occupied by alkali metals in A_xC_{60} ($x=3, 4, 6$). The conventional f.c.c. cell has been redrawn as an elongated b.c.t. cell ($c/a = \sqrt{2}$). The f.c.c. structure has one octahedral and two tetrahedral sites per C_{60} available for alkali-metal cations. In the b.c.c. structure, there are six tetrahedral sites per C_{60} , which are 31% larger than the f.c.c. tetrahedral sites. The sphere packing in the b.c.t. structure is similar to that of b.c.c., with a compression relative to b.c.c. of $c/a = 0.906, 0.921$ and 0.949 when A is K, Rb and Cs respectively. The tetrahedral sites in the b.c.t. structure are distorted from the ideal tetrahedra in b.c.c. A_6C_{60} by $\sim 2\%$. We are now conducting measurements of the physical properties of A_4C_{60} . From d.c. magnetization measurements we find no evidence of superconductivity in any A_4C_{60} sample other than that arising from an A_3C_{60} impurity.

To provide insight into the relative stabilities of the various A_xC_{60} phases, we have calculated the electrostatic energies. Table 2 summarizes the results for Rb_xC_{60} . The Madlung contribution in electron volts per excess electron was calculated with a standard computer program assuming point charges for the Rb^+ and C_{60}^{x-} ions, with experimental lattice parameters for existing phases. The electrostatic energy of putting x electrons on a C_{60} molecule was assumed to be of the form

$$E_x = -E_A + C_x/R$$

where E_A is the electron affinity of neutral C_{60} , taken to be 2.6 eV. (A change in the assumed value of E_A would alter the absolute numbers in the last column, but the energy differences would be unchanged.) The term C_x/R is the minimum coulomb repulsion of x point charges on a sphere of radius R , with R taken to be the C_{60} radius of 3.5 Å. The total calculated electrostatic energy is expressed as the energy per excess electron per C_{60} , allowing the electrostatic contributions to be directly compared. Note, first, that the three phases with lowest calculated electrostatic energy are the three experimentally observed phases. Because of the approximate method used to estimate E_x , and the neglect of van der Waals, chemical or packing interactions, the electrostatic energies of these three phases are essentially equal. The energies of these phases are, however, significantly different from the energies of the alternatives. For $x=4$, the electrostatic forces promote the distortion of the b.c.c. structure into the observed b.c.t. structure. Electrostatic forces alone, however, would result in a far larger effect. The relatively small observed difference between a and c must result from packing limitations. □

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ACKNOWLEDGEMENTS. We thank S. J. Duclos, A. F. Hebard, A. J. Muller and K. Rabe for discussions.

Off-axis orientation of the electronic transition moment for a linear conjugated polyene

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IN many applications of conjugated polyenes for nonlinear optoelectronics and as probes of biophysical systems, the orientation of the electronic transition dipole moment relative to the long axis of the chains is an important quantity. Simple models predict that the transition moment lies closely along the chain axis^{1,2} or at an angle of about 30° to this axis³, but the difficulty of preparing perfectly oriented samples has made these predictions hard to test. Here we report the results of polarized single crystal spectroscopy of a linear conjugated tetraene in the highly aligned configuration made possible by incorporating these molecules as guests in the channels of urea crystals. The angular dependence of the absorption spectrum indicates that the transition moment lies at an angle of 15° to the chain axis. Molecular-orbital calculations can reproduce this value when they include the effects of electron correlation.

It has been stated, perhaps prematurely, that if there is anything that we understand in molecular spectroscopy, it is the electronic excitations of linear conjugated polyenes⁴. These simple conjugated chains form one of the main classes of coloured compounds such as the carotenoids. The electronic transition that gives these compounds their colour is strongly allowed, and there have been many studies of its energy, strength and vibronic structure^{1-3,5-12} since the theory of the relationship between constitution and colour was first developed. There is a renewed interest in the spectroscopy of this class of compounds because of their nonlinear optical properties and because they serve as finite models for conductive polymers¹³⁻¹⁵.

One aspect of the transition that has not received much attention is the orientation of the transition dipole relative to the direction of the conjugated chain^{9,10,16,17}. Simpson³ and Parkhurst and Anex^{16,17} recognized that this transition dipole

Received 24 July; accepted 1 August 1991.

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orientation is of interest because the two simplest theories of electronic excitations in these molecules make different predictions as to its orientation. The predicted orientation is sensitive to the assumptions but not the parameters of the models, and therefore the validity of simple theories can in theory be tested by measuring the transition dipole orientation.

To determine the absolute orientation of the transition dipole between two states of a polyatomic molecule, one must either obtain and analyse a rotationally resolved absorption spectrum¹⁸ or prepare a single crystal in which the orientation of the molecule is well determined and the optical properties of the crystal are favourable. We have discovered a class of mixed crystals that are particularly well suited to the determination of the transition dipole orientation of simple alkyl-substituted linear conjugated polyene chains.

It has been known for many years that urea forms a hexagonal crystalline complex with long-chain aliphatic molecules such as *n*-alkanes and fatty acids^{19–26}. The structure of these complexes is that of a hydrogen-bonded helix of urea surrounding the long-chain compound which lies along the hexagonal *c*-axis of the crystal²⁰. For *n*-alkanes, the included *n*-alkane molecules are in a fully extended conformation. The size of the 'channel' in the hydrogen-bonded network into which an *n*-alkane fits is just large enough for the molecule, using standard van der Waals radii (Fig. 1). There is no space for angular disorder within the crystal channel. The crystallographic results have been interpreted as indicating that six orientations of the C–C plane of the alkane chain are equally probable^{20,23}.

We have found that linear conjugated polyenes that have long *n*-alkane tails on one or both ends will form stable hexagonal urea inclusion complexes²⁷. Crystals of this type can be formed in which an *n*-alkane or other nonchromophoric species is the main component and the included alkyl-polyene is a dilute species. In this way the sample can be made to have low absorbance, and exciton effects are eliminated. We have prepared such mixed crystals in which the conjugated tetraene parinaric acid (octadeca-9,11,13,15-tetraenoic acid)^{28,29} is dilute in a hexadecane/urea inclusion complex.

If the transition dipole is oriented along the polyene chain, then the absorption of these crystals will be entirely polarized

along the *c*-axis. If the transition-dipole orientation is at some angle, θ , with respect to the polyene chain axis then, because of six-fold disorder, the dipole will lie on a cone at this angle with respect to the *c*-axis. The geometry of this experiment is shown in Fig. 2. Two polarized fluorescence excitation spectra are shown in Fig. 3. As the spectrum obtained with perpendicular polarization is not negligible, the transition dipole is not strictly along the polyene chain.

We have measured the intensity of fluorescence over the whole range of angles of polarization of the incident light at both 321 nm and 306 nm. Elementary considerations of the hexagonal symmetry lead to an expression for the intensity as a function of incident polarization with the transition-dipole orientation as a parameter. Any possible overall misalignment of the crystal is included in this analysis as an angular offset. We found the off-axis angle for the transition dipole of the tetraene parinaric acid to be $20.0 \pm 1^\circ$. Measurement of the degree of polarization of fluorescence demonstrates that the transition moment for this transition is also oriented at 20° to the hexagonal axis. Time-resolved polarized fluorescence measurements in the time range 0.5–35 ns show that the fluorescence polarization is constant in time, with a value consistent with cone angle of 20° .

An independent determination of the orientation of the transition-dipole moment for the tetraene chromophore of parinaric acid has been made by analysing the time-resolved fluorescence polarization of deca-2,4,6,8-tetraene in a viscous hydrocarbon solution³⁰. The basis of this method is that if the transition dipole is at an angle to the polyene chain, then rotational diffusion around the long axis of the polyene molecule will

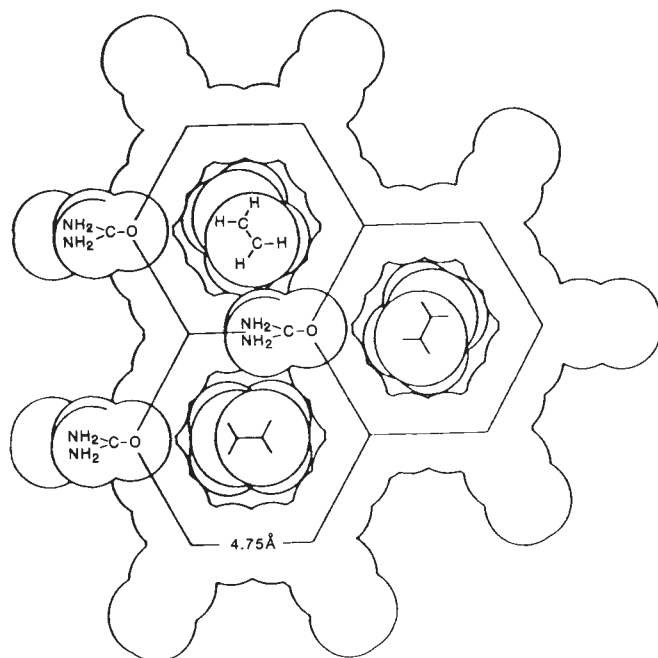


FIG. 1 Schematic illustration of the structure of the *n*-alkane/urea inclusion complex as viewed along the *c*-axis. (From ref. 26, as modified from ref. 20.)

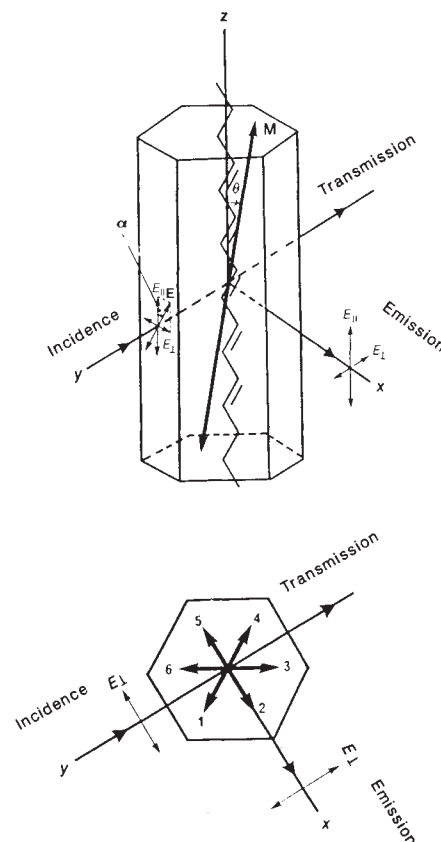
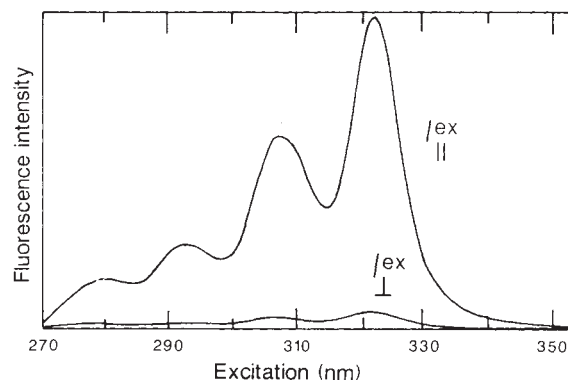


FIG. 2 Schematic illustration of the polarized single-crystal fluorescence excitation or absorption experiments used to determine the orientation of the transition dipole of a conjugated tetraene in a urea inclusion complex. **M** is the transition moment vector. The angle θ is the orientation of the transition moment with respect to the long axis of the polyene chain; α is the angle of the electric field vector, **E** of the incident radiation relative to the *c*-axis of the crystal.

FIG. 3 Fluorescence excitation spectra of parinaric acid in a urea inclusion complex. The fluorescence intensity with polarization parallel to the *c*-axis is measured for the excitation electric field oriented parallel and perpendicular to the *c*-axis of the complex, $I_{\parallel}^{\text{ex}}$ and $I_{\perp}^{\text{ex}}$ respectively.



result in a rapid depolarizing motion which can be distinguished from the slower end-over-end rotation. As for the crystal measurements, this analysis indicates $\theta = 20 \pm 1^\circ$.

To compare these measured orientations with theory, it is necessary to take into account the surrounding medium. From a continuum dielectric model for an ellipsoid of revolution^{31,32} with dimensions appropriate to that of the polyene chromophore, we conclude that the orientation of the transition moment for the isolated chromophore would be $15 \pm 1^\circ$. The uncertainty due to estimation of the cavity size is only $\sim 0.5^\circ$ (ref. 27).

The simplest model for the electronic excitations of linear polyene chains is based on the behaviour of independent electrons in a one-dimensional box of appropriate length^{1,33}. As this abstraction does not permit any off-axis transition component, its success in describing the strongly allowed transition from highest occupied molecular orbital (HOMO) to lowest unoccupied molecular orbital (LUMO), $1A_g$ to $1B_u$, of polyenes cannot be taken as indicating that the transition dipole is oriented along the long axis of the molecule. A simple model representing an opposite extreme is to consider the polyenes as a collection of coupled ethylenic units³. This excitonic model also describes the low-energy excitation of linear polyenes reasonably well. In this case the transition-dipole orientation is predicted to be along the ethylenic units, corresponding to $\theta = 30^\circ$. This value is independent of the polyene chain length for the simplest version of the model.

Another simple picture of polyene excitations is provided by Huckel molecular orbital theory. In this case the transition dipole orientation depends on the ratio of the exchange integrals chosen for the consecutive bonds of the polyene chain and also depends on the chain length. A higher degree of bond alternation results in a larger off-axis angle with an upper limit of 30° . Adjusting the parameters of this model does not result in satisfactory agreement with experiment² unless different parameter values are adopted for polarization and excitation energy data³⁴.

The simple Huckel molecular orbital treatment ignores electron correlation except in so far as it is indirectly involved in determining the bond alternation. Treatments that explicitly include electron-electron interaction in a semiempirical π -electron approximation have been shown^{10,35} to predict a change in the ordering of the low-lying excited electronic states when doubly and more highly excited configurations are included in the calculation. The $1B_u$ excited electronic state remains essentially a singly excited HOMO to LUMO excitation in these treatments. On this basis, one would not expect the inclusion of electron correlation to influence the polarization of the transition moment. But it must be noted that the ground electronic state is considerably changed by the inclusion of doubly excited configurations. This effect has not yet been systematically analysed. The polarization direction expected on the basis of a complete Pariser-Parr-Pople calculation with complete π -configuration interaction for octatetraene has been reported by Ramasesha and Soos³ as 14.9° , in good agreement with our

experimental value. We tentatively conclude that including electron correlation a π -electron treatment is necessary and sufficient to characterize the transition-moment orientation of linear polyene chains, which is intermediate between the predictions of the simplest uncorrelated and highly correlated descriptions.

The orientation of the transition for the strongly allowed electronic excitation of linear polyene chains is of practical importance in the interpretation of experiments concerning the dynamics of such molecules when they are used as a probe species in biophysical experiments^{28,29}. Specifically, the fact that the transition has an off-axis orientation means that the behaviour of the anisotropy at very short times will include components from axial motion. The orientation is also important in determining the magnitude of the off-axis tensor components of nonlinear optical susceptibilities^{13-15,37}. □

Received 15 April; accepted 1 July 1991.

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ACKNOWLEDGEMENTS. We thank D. Daikh, T. Nestor, K. Zabel, V. Lambert, T. Novet, S. Lee and I. Johnson, who performed some of the initial experiments, and P. Engelking for a discussion on the cavity correction. This research was supported by the US Office of Naval Research and by the NSF.

Reconstruction of past changes in salinity and climate using a diatom-based transfer function

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THE prospect of global warming has focused attention on the role of palaeoecology in testing the accuracy and sensitivity of climate-model predictions, in identifying past analogues for future climate change, and in placing model-predicted climate responses in the context of natural climate variability^{1,2}. Proxy data for climate reconstruction can be derived from many sources, including the palaeolimnological record^{3,4}. In closed-basin lakes in arid and semi-arid regions, shifts in effective moisture lead to the concentration or dilution of dissolved salts, and these changes in salinity are clearly reflected in the composition of lacustrine diatom assemblages⁵⁻⁸. Here we refine a previously published⁹ diatom-based transfer function for the reconstruction of past changes in salinity of lakes in the northern Great Plains region of North America, and apply the refined transfer function to a late-glacial and Holocene sediment record from Devils Lake, North Dakota. Our results show that there were a number of alternations between fresh and saline conditions during the Holocene and hence demonstrate the utility of the technique in reconstructing past changes in regional climate.

Our transfer function for salinity reconstruction is based on the statistical relationship between regional modern diatom assemblages in surface sediments and lakewater chemistry. We collected surface-sediment samples for diatom analysis and associated data on water chemistry in 1982 and 1985 from 39

sites in North and South Dakota, USA and from 27 lakes in Saskatchewan, Canada. The lakes had salinity ranging from 0.7‰ to 270‰ and the sites had a strong gradient from east to west in their precipitation minus evaporation gradient (Fig. 1). Standard techniques were used for diatom analysis¹⁰. In most cases diatom counts were 400–600 valves, but in samples where diatoms were scarce or poorly preserved, fewer were counted. Cation concentrations were measured by d.c.-plasma atomic emission spectrometry, anions by ion chromatography and inorganic carbon by automated coulometric titration. Statistical analysis of the data on water chemistry and diatoms was carried out using principal-component analysis, detrended correspondence analysis and canonical correspondence analysis¹¹⁻¹³. In all ordinations the first axis was strongly related to total salinity and many of its associated ionic components, and the second axis related to differences in brine type, in particular the difference between systems dominated by CO₃/HCO₃ and those dominated by SO₄. The combined data set was screened, and 11 samples where diatoms were absent or poorly preserved were omitted from further analysis.

On the basis of the strong first-axis relationship between diatom composition and salinity, we used weighted-averaging regression¹⁴ of the log-transformed salinity data to estimate the salinity optima and tolerances of all taxa that occurred with at least 2% abundance in any one sample. We subsequently used weighted-average calibration with an inverse deshrinking regression¹⁵ to estimate the diatom-inferred modern salinity for each site in the data set. A previously published transfer function⁹, derived from canonical correspondence analysis¹⁶, was based on classical regression, which produces larger root-mean-square errors and pulls inferred values away from the mean¹⁵. Optima

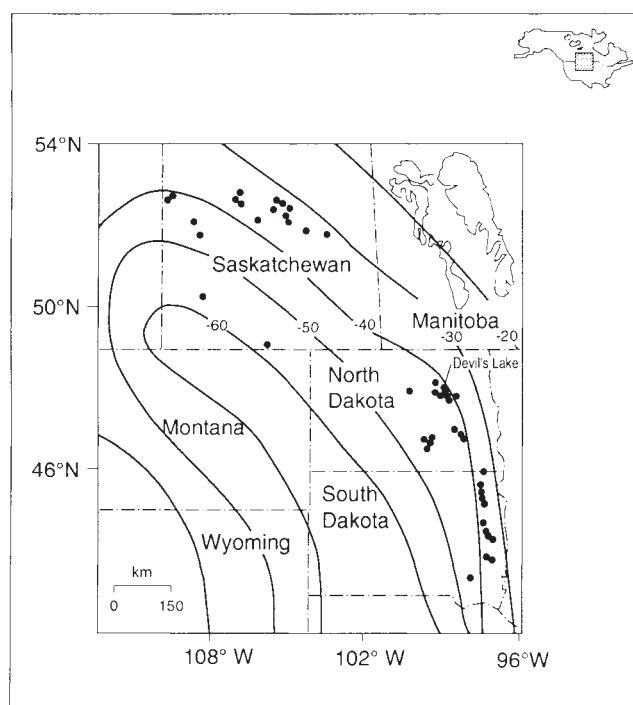


FIG. 1 Map of the northern Great Plains showing the location of surface-sample sites and Devils Lake. The contours are lines of equal precipitation minus evaporation, measured in cm yr⁻¹ (ref. 18).

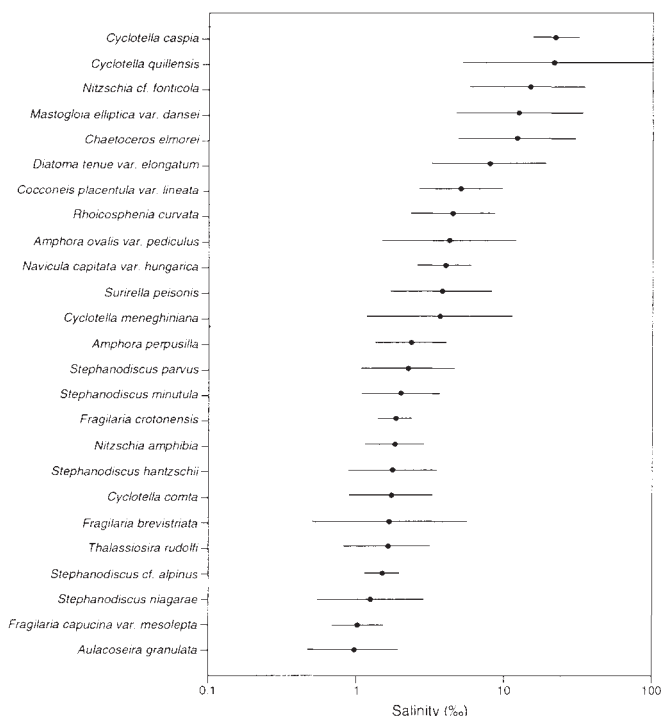


FIG. 2 Estimated optima (abundance-weighted means) and tolerances (abundance-weighted standard deviations) of taxa in the Devils Lake core with maximum abundance >5% and occurrences in five or more samples.

$$\hat{u}_k = \frac{\sum_{i=1}^m y_{ik} x_i}{\sum_{i=1}^m y_{ik}} \quad \hat{t}_k = \left[\frac{\sum_{i=1}^m y_{ik} (x_i - \hat{u}_k)^2}{\sum_{i=1}^m y_{ik}} \right]^{1/2}$$

where y_{ik} is the abundance of taxon k in sample i , x_i is the observed salinity of sample i , and $\hat{u}_k$, $\hat{t}_k$ are the optimum and tolerance of species k , respectively.

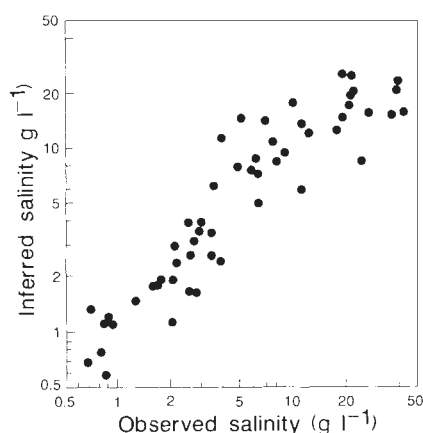


FIG. 3 Observed salinity against diatom-inferred salinity (derived from weighted-averaging regression) for the 55 surface-sample sites ($r=0.91$).

$$\hat{x}_i = \frac{\sum_{k=1}^m y_{ik} \hat{u}_k}{\sum_{k=1}^m y_{ik}} \quad \hat{x}_i = a + b \hat{x}_i$$

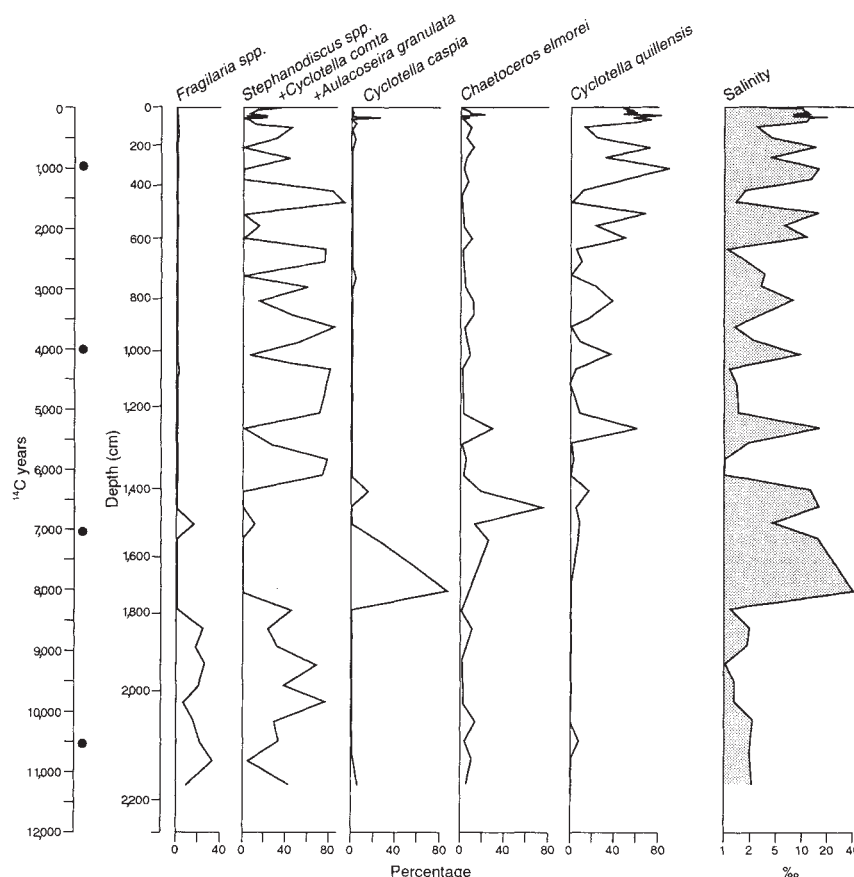
where y_{ik} is the abundance of taxon k in sample i , $\hat{u}_k$ is the optimum of species k , $\hat{x}_i$ is the inferred salinity, and a and b are the coefficients of the deshrinking regression of observed salinity x_i on $\hat{x}_i$ (ref. 72 in ref. 15).

and tolerances of selected diatom taxa and a comparison between inferred and measured salinity for each site are shown in Figs 2 and 3, respectively. The data indicate a very close agreement between inferred and measured salinity with no apparent outliers, despite the variable state of diatom preservation in the surface-sediment samples. These results suggest that diatom preservation is not significantly biased towards either the fresh or saline ends of the spectrum or that differential loss of entire valves is small.

To evaluate the use of the transfer function, we took cores from Devils Lake, North Dakota (48°05' N 98°56' W), where historical records show a strong hydrological response to climate change in the past century¹⁷. Lake levels fell and salinity rose from the late nineteenth century through the drought years of the 1930s and 1940s. Thereafter levels rose and salinity decreased to the present day (modern salinity is 2.8‰). This cycle of

water-level change is recorded in the uppermost 30 cm of sediment by changes in the diatom composition. The recent fresh-water phases are characterized by *Stephanodiscus minutulus* Kütz. Cleve and Moller and *S. niagarae* Ehr., whereas *Cyclotella quillensis* Bailey and resting spores of *Chaetoceros elmori* Boyer dominate earlier saline periods. The highly saline low-water stands of the 1930s to 1940s also contain increased percentages of benthic diatoms and the meso-polysaline taxon *Cyclotella caspia* Grun. Application of both the preliminary⁹ and the revised transfer functions to this time period showed that the salinity history could be accurately reproduced for periods of fresh water and low salinity (<10‰) and that high-salinity intervals could be clearly distinguished from those of low to moderate salinity. Discrepancies between the measured and diatom-inferred salinity during high-salinity episodes for Devils Lake probably result from problems with the sedimentary

FIG. 4 Summary diatom diagram and diatom-inferred salinity for the late-glacial and Holocene record of Devils Lake, North Dakota. Core depth is measured from the sediment surface. Dates on the y-axis are derived from an age-depth relationship based on four AMS radiocarbon dates: Beta-21193/ETH-3022, 108–116 cm (woody fragment), 955 ± 120 BP; beta 21023/ETH-3009, 995–1003 cm (woody fragment), $3,950 \pm 150$ BP; beta-20471/ETH-2925, 1507–1515 cm (woody fragment), $6,855 \pm 110$ BP; beta-20472/ETH-2926, 2131–2139 cm (conifer twig), $10,580 \pm 160$ BP. The dating uncertainty limits represent 1 s.d. from counting statistics; dates are corrected by ^{13}C for total isotope effects; •, position of dates.



record, such as poor diatom preservation, sediment mixing and reworking, or dating inaccuracies, rather than from inadequacies in the inference method itself.

Diatom analysis of a 24-m-long, ^{14}C -dated by accelerator mass spectrometry (AMS) core from Devils Lake (Fig. 4) showed that the late-Wisconsin/early-Holocene was characterized by freshwater taxa, such as *S. minutulus*, *S. niagarae*, *Cyclotella comta* (Ehr.) Kütz. and *Fragilaria* sp. These freshwater species were abruptly replaced $\sim 8,000$ yr BP by *Cyclotella caspia* and other euryhaline species indicating a phase of low water level and high salinity that persisted until $\sim 7,000$ yr BP. A series of oscillations between fresh and saline episodes then followed, as shown by the alternation of freshwater *S. niagarae*, *S. minutulus* and *Aulacoseira granulata* (Ehr.) Ralfs. with the saline *C. quillensis* and *C. elmorei*.

Application of the transfer function to the Holocene stratigraphy suggests that salinities in the lake have fluctuated from ~ 1 to 40‰, with highest salinities in the early Holocene (Fig. 4). These oscillations in salinity, if repeated synchronously at other sites in the region, suggest that maximum aridity occurred $\sim 8,000$ yr BP, and that effective moisture fluctuated cyclicly throughout the remainder of the Holocene. At least seven oscillations are indicated by the Devils Lake data. The data further suggest that the lake was much more saline and conditions much drier in the past than the present day.

Because of high rates of sediment accumulation in many endorheic basins and a rapid chemical response to hydrological change, the diatom stratigraphy of saline-lake sediments can provide a sensitive, high-resolution record of climate change, without the lags characteristic of many other palaeoclimate proxies. We still need to extend our calibration data set at both ends of the salinity gradient to describe the weighted-averaged salinity optima of some taxa more accurately and to assess further the importance of variable diatom preservation in both the calibration data set and the core samples. Moreover, the relationship of salinity to water level and climate is complex and varies from lake to lake depending on hydrological and morphometric features of the lake and its watershed, as well as on geochemical characteristics of the lake water. For the northern Great Plains, where greenhouse warming could have a considerable impact on water availability and the agricultural economy, a series of accurately dated cores from additional sites are needed to substantiate the inferences that we have drawn here about effects on the climate. We can then use the pattern of events thus indicated to validate and refine moisture simulations from general circulation models, and to predict regional responses to climate change. □

Nanometre-size diamonds in the Cretaceous/Tertiary boundary clay of Alberta

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STARTING with the discovery of an iridium anomaly at the Cretaceous/Tertiary boundary in Italy¹, the idea that a large asteroid or comet struck the Earth at the end of the Cretaceous period has gained wide acceptance²⁻⁴ although some workers suggest that massive volcanic eruptions can also explain the observations⁵. The abundance of small diamonds, 3–5 nm in size, in chondritic meteorites^{6,7} prompted us to search for such diamonds in the Cretaceous/Tertiary boundary clay of the Red Deer Valley of Alberta (the 'Knudsen's farm' locality). Dissolution and oxidation of this clay yielded 45 ng g⁻¹ of a white fraction, consisting of more than 97% carbon, which was absent from rocks above and below the boundary layer. We present evidence that this material is indeed diamond, which strengthens further the case for an extraterrestrial impact. The diamond/iridium ratio in the boundary clay may constrain the type of impactor; our estimate (1.22:1) is close to the value found in type C2 chondritic meteorites².

The stratigraphy and palaeontology of the 'Knudsen's farm' section, located along the Red Deer Valley (51° 54' 10" N, 113° 00' 50" E) in Alberta, Canada, have been much studied^{4,8-13}. The K/T boundary lies within the Scollard formation, the sediments of which were laid down in terrestrial, not marine environments. The 1-cm-thick boundary claystone overlies many metres of sandstones and mudstones and is capped by a coal seam half a metre thick, followed by more mudstones, both with Tertiary microflora. The sampled boundary clay is a medium grey claystone and is equivalent to the 'fireball layer' of the compound boundary layer described by Hildebrand and Boynton⁴.

Following the procedure adopted by Lewis *et al.*¹, we dissolved the major minerals from a 20-g sample with HF and HCl. The residue, consisting of 114 mg of solids, was suspended in distilled water and the supernatant colloid decanted off. After washing and repeated decanting, the discarded residue consisted chiefly of spinel and chromite. Spinel and chromite have previously been noted in the boundary claystone at other localities¹⁴⁻¹⁶. The colloidal supernatant fraction was oxidized by successive treatment with 20 M HNO₃ (16 h at 70 °C) and concentrated HClO₄ (2 h at 140 °C). This is adequate to dissolve amorphous carbon and graphite as well as coal macerals¹⁷. A final washing with HF and HCl, to remove any remaining silicates, yielded 0.90 mg of a white residue. On the unlikely supposition that this represents almost 100% recovery from the original 20-g sample then the concentration in the rock is at least 45 ng g⁻¹, or, integrated over the rock section, 129 ng cm⁻².

Energy-dispersive X-ray analysis of the white residue fraction in the scanning electron microscope showed no element of higher mass than silicon. Magnesium, aluminium and silicon together accounted for $\sim 2\%$ of the total mass with the remainder composed of carbon. X-ray diffraction showed three broad diffuse lines which matched the three principal lines of diamond. In the transmission electron microscope, the grains were found to be $\sim 3\text{--}5$ nm in size and, wherever any morphology could be discerned, octahedral in form, rather than the hexagonal plates of graphite. Electron diffraction (ring powder) gave spacings characteristic of diamond: 2.06, 1.26, 1.08, 0.891, 0.819, 0.73 and 0.68 Å (Fig. 1). We conclude that this material is indeed diamond.

Similar samples prepared from the coal seam 2 cm above the boundary clay, and from the mudstones 3 cm below and 1 m

Received 22 February; accepted 2 July 1991.

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ACKNOWLEDGEMENTS. We thank E. Almgren, G. Battarbee, S. Björck, D. Haviland, H. Jacobson, S. Patrick, G. O. Seltzer, J. A. Wolin and H. E. Wright Jr for assistance with field work. This study was funded in the USA by the NSF (Ecology Program) and in the UK by the NERC (Palaeoclimate Special Topic).

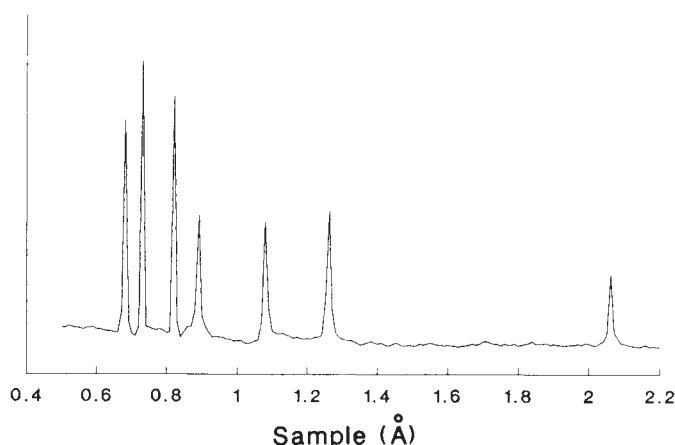


FIG. 1 Electron diffraction profile of the powder. The peaks correspond closely to the accepted values for diamond.

above yielded no residue, not even spinel or chromite, when extracted by the same process. We conclude that the white residue which survives the extraction procedure is confined to the boundary clay itself. This diamond fraction is distinct from the abundant soot found at the K/T boundary¹⁸.

Badziag *et al.*¹⁹ have proposed that, under conditions pertaining in the early Solar System, the energetically preferred form of elemental carbon is diamond, in the 3–5-nm size range discussed here, rather than graphite. Correspondingly, Huss⁷ has shown that diamonds were primitively incorporated into all chondritic meteorite groups at concentrations of 500–1,000 $\mu\text{g g}^{-1}$. In addition, spinel and chromite are commonly found in chondrites^{6,14–16}, and they too were present in the boundary layer but absent above and below. Impact by one or more meteorites or comets, a possible cause for the event horizon at the K/T boundary which has strong support², may therefore be expected to distribute a diamond powder along with the other debris. The diamond powder is indeed present in the boundary clay, but absent a few centimetres above or below. The powder cannot have originated in volcanic action because the micro-diamonds would have reverted to graphite or oxidized to carbon-dioxide at the temperatures and relatively low pressures characteristic of volcanic eruptions, nor can it have arrived directly from a supernova, for the diamonds would have burned up as micrometeorites in the upper atmosphere. The possibility remains that the diamonds may have arisen by shock metamorphism of carbonaceous target rocks at the site of an impact. In principle, a good X-ray diffraction photograph could allow us to determine whether the diamonds were shock-produced, but the quality of our photographs is not adequate for this. The question of terrestrial or extraterrestrial origin can, however, best be addressed by an examination of isotope ratios, which we intend to study next.

In the meantime we note that the ratio of diamond to iridium (1.22:1) is close to that found in C2 chondritic meteorites². Integrated over the rock column, the concentration of diamonds is 129 ng cm^{-2} , and that of iridium, which is smeared over a greater depth of the boundary rocks, may be estimated at 105 ng cm^{-2} (our own data), giving a diamond/iridium ratio of 1.22:1. The diamond concentration of the C2 chondritic meteorites Murrumbidgee and Murchison has been variously estimated^{6,7} at 360 to 800 p.p.b., and that of iridium has been reported^{1,2} at 300 to 650 p.p.b. Taking the ratio of the extremes of these measurements, we may estimate the diamond/iridium ratio in these C2 chondritic meteorites at 360:300 and 800:650 respectively, or 1.20:1 and 1.23:1 (ref. 2). □

Received 15 March; accepted 25 June 1991.

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ACKNOWLEDGEMENTS. We thank K. Knudsen for permission to access the sampled rock section, the late P. Weinberger for her assistance with this work and for access to equipment, and T. R. Carlisle for help in field work.

Evolutionary distinctiveness of the endangered Kemp's ridley sea turtle

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THE endangered Kemp's ridley sea turtle (*Lepidochelys kempi*) nests almost exclusively at a single locality in the western Gulf of Mexico, whereas the olive ridley (*L. olivacea*) nests globally in warm oceans. Morphological similarities between *kempi* and *olivacea*, and a geographical distribution that "... makes no sense at all under modern conditions of climate and geography"¹, raise questions about the degree of evolutionary divergence between these taxa. Analysis of mitochondrial (mt) DNA restriction sites shows that Kemp's ridley is distinct from the olive ridley in matriarchal phylogeny, and that the two are sister taxa with respect to other marine turtles. Separation of olive and the Kemp's ridley lineages may date to formation of the Isthmus of Panama, whereas the global spread of the olive ridley lineage occurred recently. In contrast to recent examples in which molecular genetic assessments challenged systematic assignments underlying conservation programmes^{2–6}, our mtDNA data corroborate the taxonomy of an endangered form.

From >40,000 adult females censused in a single mass nesting event in the late 1940s, the number of Kemp's ridley turtles nesting annually at the primary site in Tamaulipas, Mexico, has declined dramatically to only a few hundred in recent years^{7–9}. Kemp's ridley, first recognized in 1880 (ref. 10), has had a troubled taxonomic history. Before the discovery of the principal nesting site, Kemp's ridley was commonly known as the bastard loggerhead, reflecting belief that it was a hybrid between the loggerhead (*Caretta caretta*) and either the hawksbill (*Eretmochelys imbricata*) or the green (*Chelonia mydas*) turtle¹¹. Early in this century, some authors classified the Kemp's ridley as a loggerhead (*Caretta kempii*)¹² or as a subspecies of the olive ridley (*L. olivacea kempii*)¹³. A further complication has been that the Kemp's ridley is routinely misidentified as a loggerhead in museum collections. *L. kempi* is restricted in range to the Gulf of Mexico and the north Atlantic. *L. olivacea* occurs in the east and west Pacific, Indian Ocean, and both sides of the Atlantic, but does not overlap geographically with the Kemp's ridley¹⁴.

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TABLE 1 Description and distribution of mtDNA genotypes in ridley and representative loggerhead turtles

Taxon	Number of turtles	mtDNA genotype																
<i>L. kemp</i>	4	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C
<i>L. kemp</i>	2	C	C	C	C	C	C	C	C	C	C	C	C	C	B	C	C	C
<i>L. olivacea</i>	19	B	E	E	C	D	C	B	C	C	C	D	C	B	D	C	B	C
<i>C. caretta</i> (Atlantic)	5	—	E	—	D	—	—	E	B	E	D	A	C	D	—	C	—	E
<i>C. caretta</i> (Pacific)	5	—	E	—	E	—	—	E	B	F	D	A	C	E	—	C	—	E

Letters refer to mtDNA digestion profiles produced by (from left to right) *Avall*, *BclI*, *BstEII*, *BstNI*, *Clal*, *EcoRI*, *EcoRV*, *EcoO109*, *HincII*, *HindIII*, *NdeI*, *PvuII*, *SacI*, *SpeI*, *SstII*, *StuI* and *XbaI*: adjacent letters in the alphabet indicate that fragment profiles differ by a single restriction site; nonadjacent letters differ by at least two sites. Dashes indicate enzymes for which site comparisons were not made between loggerhead and ridley.

The international programme to protect Kemp's ridley represents the largest conservation effort for any marine turtle¹⁵. As in other conservation programmes, exceptional preservation efforts are motivated ultimately by the premise that the endangered population is a distinct phylogenetic entity. Here we use conventional mtDNA restriction site analyses to elucidate evolutionary relationships and phylogeny within *Lepidochelys*. Mitochondrial DNA from Kemp's ridley ($n = 6$) was compared with that from two widely separated populations of olive ridley (Suriname, West Atlantic, $n = 14$; Costa Rica, East Pacific, $n = 5$) after digestion with the 17 restriction endonucleases listed in Table 1. Loggerhead turtles were included as an outgroup, based on an accepted subfamily affiliation¹⁶.

A mean of 80 restriction sites per individual, corresponding

to 461 nucleotides in recognition sequence, was scored in the ridleys (Fig. 1). Two genotypes (separated by a single restriction enzyme site change) were observed in the Kemp's and one in the olive ridley. The latter genotype differed from those in the Kemp's ridley at 8–9 of the 17 digestion profiles (Table 1), involving changes at 10–11 assayed restriction sites. Genetic distances are summarized in Table 2.

Phylogenetic summaries of mtDNA data (Fig. 2) are consistent with the current morphology-based taxonomy of the Kemp's and olive ridley. Atlantic and Pacific populations of the olive ridley, separated by about 25,000 km of ocean, were indistinguishable in our assays (sequence divergence, $p = 0.000$), whereas the Kemp's ridley showed substantial mtDNA differences from both ($p = 0.012 \pm 0.003$ (s.e.; ref. 17)). The mtDNA sequence divergence between Kemp's and olive ridleys is also greater than any estimated genetic distances in global surveys of either the green or loggerhead turtle (Fig. 2). On the basis of a provisional mtDNA clock calibrated from other marine turtles (0.2 to 0.4 per cent sequence divergence per million years (see legend to Fig. 2)), we estimate that the olive and Kemp's ridleys diverged about 3–6 million years ago, whereas the two widely separated olive ridley populations diverged recently. Although absolute time estimates must be interpreted with caution, mtDNA data are consistent with a suggestion that Kemp's and olive ridley were isolated by formation of the Isthmus of Panama¹⁸ some three million years ago.

Molecular data are also consistent with the hypothesis that olive ridley populations in the Atlantic and Pacific Oceans are closely related¹⁴. Based on a synthesis of distributional data and comparative morphology, Pritchard¹⁹ suggested that olive ridleys recently colonized the Atlantic via the Cape of Good Hope. Contemporary oceanic current patterns and the presence of olive ridleys in southeast Africa are both compatible with this view²⁰, and this route has been widely cited as a path of Atlantic colonization by other Indo-Pacific faunas²¹. Our data support Pritchard's¹⁹ biogeographic scenario for the olive ridley, but a complete test should include additional population samples from spatially intermediate locales.

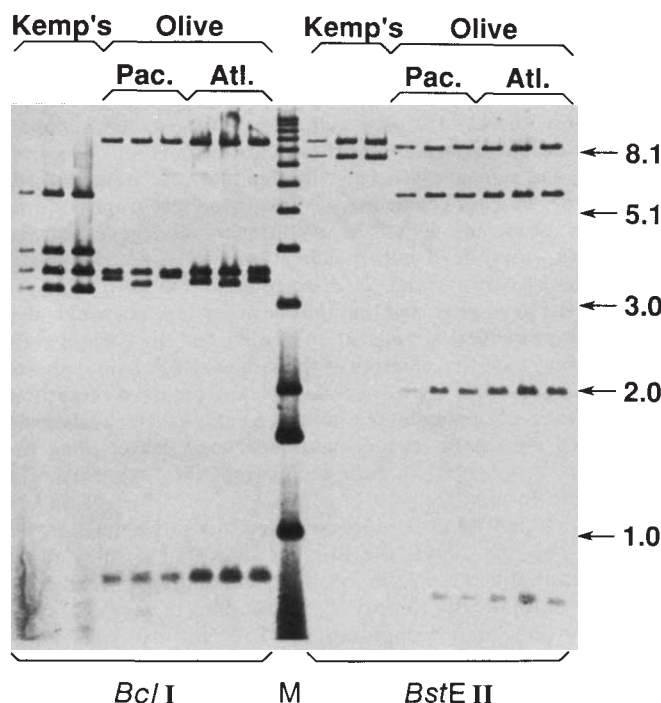


FIG. 1 Examples of mtDNA digestion profiles (produced by *BclI* and *BstEII*) that are identical in olive ridley populations from the Atlantic (Atl.) and Pacific (Pac.) Oceans, but which each distinguish Kemp's from olive ridley by two restriction-site changes. Heart and liver samples of *L. kemp* were obtained from cold-stunned juveniles that perished near Long Island, New York from 1987–1989. Samples of *L. olivacea* consisted of eggs (one per female) taken during laying and incubated in the laboratory for two to eight weeks. MtDNA was isolated by CsCl density-gradient centrifugation²⁵. Restriction fragments were end-labelled with ³⁵S-labelled radionucleotides, separated on 1.0–1.5% agarose gels and visualized by autoradiography²⁵. In this gel the centre lane (M) is a molecular size standard; selected fragment sizes (in kilobases) are indicated to the right. Note that in olive ridleys, the *BclI* fragments near 3.3 kb also exhibit a size polymorphism (as judged by concordant differences in digestion profiles from other enzymes).

TABLE 2 Estimates of mtDNA nucleotide sequence divergence based on restriction site and fragment comparisons²²

	<i>L. kemp</i>	<i>L. kemp</i>	<i>L. olivacea</i>	<i>C. caretta</i> (Atlantic)	<i>C. caretta</i> (Pacific)
I <i>L. kemp</i>	—	0.001	0.011	0.038	0.042
II <i>L. kemp</i>	0.001	—	0.012	0.040	0.039
III <i>L. olivacea</i>	0.013	0.012	—	0.036	0.035
IV <i>C. caretta</i> (Atlantic)	0.043	0.043	0.042	—	0.008
V <i>C. caretta</i> (Pacific)	0.044	0.043	0.042	0.007	—

Estimates from restriction sites (above diagonal) are based on the enzymes listed in Table 1; those from fragment comparisons (below diagonal) are based on data from *BclI*, *BglI*, *BstEII*, *BstNI*, *EcoRV*, *EcoO109*, *HincII*, *HindIII*, *NdeI*, *PvuII*, *SacI*, *SpeI*, *SstII*, *StuI* and *XbaI*.

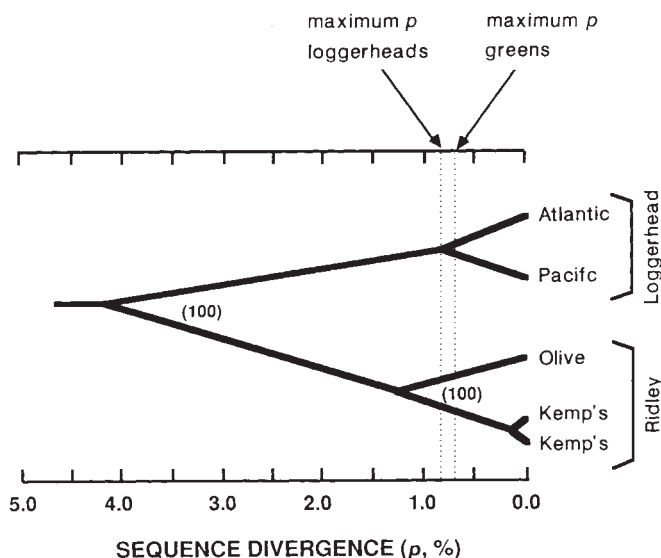


FIG. 2 UPGMA phenogram²⁶ summarizing mtDNA relationships among ridley and representative loggerhead turtles from Cumberland Island, Georgia, USA and Mon Repos, Queensland, Australia. An exhaustive computer search (using PAUP; ref. 27) identified a single most-parsimonious network, with topology identical to that of the phenogram. Among 1,000 bootstrap replicates, support for putative clades (expressed in %) are in parentheses. Sequence divergence, p , can be related tentatively to absolute time by assuming 0.2–0.4% sequence divergence between two lineages per million years. This calibration comes from analyses of several other turtle species (ref. 28, and our unpublished data), and represents a 5- to 10-fold deceleration compared with the 'conventional' vertebrate mtDNA clock²⁹. Also indicated are largest mtDNA genetic distances observed among global collections of 213 green and 144 loggerhead turtles (ref. 28, and our unpublished data).

Lepidochelys kempi and *L. olivacea* are related more closely to one another in the mtDNA tree than either is to the loggerhead (Fig. 2). Using the 'site' and 'fragment' methods²², intergeneric comparisons yielded estimates of sequence divergence (p) ranging from 0.035–0.044 (Table 2). Applying the aforementioned clock calibration, ridley and loggerhead ancestors seem to have diverged about 10–20 million years ago.

Under the terms of the US Endangered Species Act and parallel international regulations, legal protection may be extended to 'distinct' species, subspecies and populations, with the definition of these categories left to the purview of biologists and legal experts⁵. Phylogeographic data alone cannot establish whether the allopatric *kempi* and *olivacea* are isolated by intrinsic reproductive barriers, and hence qualify as distinct species under a biological species definition^{23,24}. Nonetheless, the molecular analysis demonstrates that Kemp's ridley is phylogenetically distinct from assayed olive ridley populations, with mtDNA genetic distances greater than those typically observed among global populations of other marine turtle species. □

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ACKNOWLEDGEMENTS. For field work, logistic support and permits, we thank F. Baal, M. Ball, V. Burke, R. Carlson, D. Carr, S. Cornelius, G. Cruz, J. Frazier, S. Karl, C. Limpus, L. Mendez, K. Mohadin, S. Morreale, W. Nelson, E. Possardt, H. Reichart, J. Richardson, D. Robinson, P. Ross, S. Sadove, K. Scribner, J. Woody, the Caribbean Conservation Corporation, the Servicio de Parques Nacionales de Costa Rica, the Queensland National Parks and Wildlife Service, the Okeanos Ocean Research Foundation, the Suriname Forest Service (STINASU), the US Fish and Wildlife Service, and the World Wildlife Fund. This work was supported by grants from the NSF and the National Geographic Society.

Regulation of fast inactivation of cloned mammalian $I_K(A)$ channels by cysteine oxidation

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MODULATION of neuronal excitability by regulation of K^+ channels potentially plays a part in short-term memory¹ but has not yet been studied at the molecular level. Regulation of K^+ channels by protein phosphorylation^{2–5} and oxygen⁶ has been described for various tissues and cell types; regulation of fast-inactivating K^+ channels mediating $I_K(A)$ currents has not yet been described. Functional expression of cloned mammalian K^+ channels^{7–13} has provided a tool for studying their regulation at the molecular level. We report here that fast-inactivating K^+ currents mediated by cloned K^+ channel subunits derived from mammalian brain expressed in *Xenopus* oocytes are regulated by the reducing agent glutathione. This type of regulation may have a role *in vivo* to link metabolism to excitability and to regulate excitability in specific membrane areas of mammalian neurons.

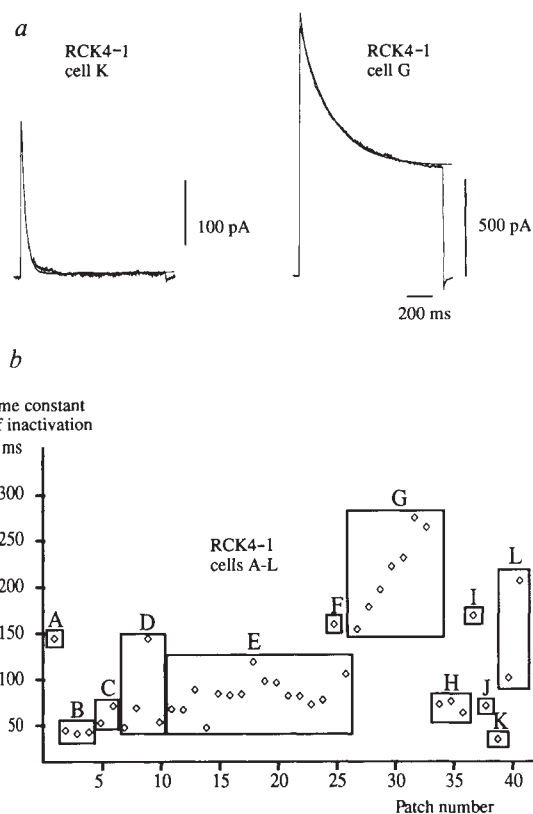
Three types of cloned rat brain $I_K(A)$ channels have been described based on K^+ channel proteins RCK4 (ref. 9), Raw3 (ref. 14) and the heteromultimer composed of RCK1 and RCK4 (RCK1,4; ref. 15). The time constants of inactivation observed for these three channel types exhibited an unusually wide scatter^{14,15}. Figure 1a compares current traces from two *Xenopus* oocytes (cell K and cell G) expressing RCK4–1 tandem channels (described in Fig. 1 legend). They had markedly different time constants (by a factor of eight) of inactivation and a different percentage of steady-state current. As shown in Fig. 1b, the value of the time constant tends to be similar for patches from any oocyte. This suggests that inactivation might be regulated by an intracellular factor common to all patches on a particular oocyte.

Received 29 April; accepted 3 July 1991.

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FIG. 1 Time course of inactivation varies among oocytes expressing the same cloned K^+ channel RCK4-1. RCK4-1 tandem channels were expressed after injection of complementary RNA transcribed from a cDNA encoding a fusion protein of one RCK4 and one RCK1 subunit. RCK4-1 tandem channels had identical kinetics and single channel properties, with the heteromultimeric channels (RCK1,4) forming spontaneously after coexpression of RCK4 and RCK1 (not shown). RCK4-1 tandem channels were preferred because they are extremely highly expressed in *Xenopus* oocytes (usually up to several hundreds of microamperes of whole-cell current). K^+ outward currents were measured in cell-attached patches in response to depolarizing test pulses. Test pulses of 1.2 s went from -80 to 40 mV. The pipette solution contained (in mM): NaCl (115), KCl (2.5), $CaCl_2$ (2), HEPES (10), pH 7.2 with NaOH. **a**, Comparison of the time courses of the fastest and of the slowest inactivation found in 41 cell-attached patches on 12 oocytes. The fastest decay (left trace) measured on cell K had a time constant of 35 ms. At the end of the trace the current had decreased to zero. The slowest decay (right trace) measured on cell G had 271 ms when fitted with one or 61 ms, and 326 ms when fitted with two exponentials. The current did not decrease to zero, leaving an offset of 40% of the peak amplitude at the end of the trace. **b**, Results of monoexponential fits of the decay time constant measured in 41 patches on 12 oocytes (A-L). Patches measured on the same oocyte are surrounded by a box marked with a capital letter designating a particular oocyte. Time constants of inactivation were correlated with individual oocytes; for example, for cell G this was 202 ± 56 ms, and 82 ± 17 ms for cell E. Cell G showed a time-dependent increase in its time constants during the 2-h recording.

METHODS. A deletion (nucleotides -492 to $1,228$ in the sequence of RCK4 (ref. 9)) in RCK4-pAKS2 (ref. 21) was the first step in the construction of the RCK4-1 tandem. Behind this partial RCK4, a *Bam*HI/*Asp*718 fragment containing the whole coding region (-492 to $1,645$ (ref. 22)) of RCK1 was cloned to generate pR4_E/R1. To connect the RCK4 with the RCK1 coding region, we used the polymerase chain reaction (PCR): the first PCR amplified the region corresponding to the C terminus of the RCK4 protein and introduced a unique restriction site (*Aat*II); in the second PCR the DNA region corresponding to the N terminus of RCK1 was amplified and the same unique restriction site was then introduced. The primers for the first PCR were 5'-AAGACGTCAGTCTCC-ACAGCCTTTGC-3', and 5'-GTCCCAGTCAAGCACTC-3', and for the second PCR, 5'-AAGA-CGTCTCAGGGGAGAATGCAGACAG-3' and 5'-GTATCCCACAGTGGTCATGGA-3'. The template (1 fmol) for both PCRs (run for 1 min at 94°C , then for 1 min at 51°C , and 1.5 min at 72°C) were *Eco*RI-linearized RCK4-pAKS2 and RCK1-pAS18 (ref. 7). The product of the first PCR was cut with *Xba*I and *Aat*II and the product of the second with *Aat*II and *Cla*I. These fragments were cloned together into a *Xba*I/*Cla*I-cut pR4_E/R1. Therefore in the RCK4-1 tandem the RCK4 protein sequence ends with TDV (single-letter amino-acid code) and starts at amino-acid number 5 in RCK1. To complete the coding sequence of RCK4, a *Hind*III/*Acc*I (nucleotides -256 to 557) fragment and an *Acc*I/*Xba*I (nucleotides 557 to $1,839$) fragment of RCK4-pAKS2 were cloned in the intermediate construct. All manipulated sequences were checked by sequencing. Capped runoff cRNA of RCK4, Raw3, *ShA*2 and RCK4-1 was synthesized as described⁷ and injected into *Xenopus* oocytes. Patch-clamp recordings were made 2-7 days after injection.



Pipettes made from thick-wall borosilicate glass had a resistance of $4\text{ M}\Omega$ and a tip diameter of $2\text{ }\mu\text{m}$. Currents were recorded with an EPC9 amplifier (Heka Electronic). Leakage and capacitive currents were measured and compensated linearly with the EPC9 at -80 mV membrane potential before the first test pulse was applied. Leakage was compensated before each test pulse. Evaluation of data and fitting of exponentials was done off-line with an IBM AT Computer.

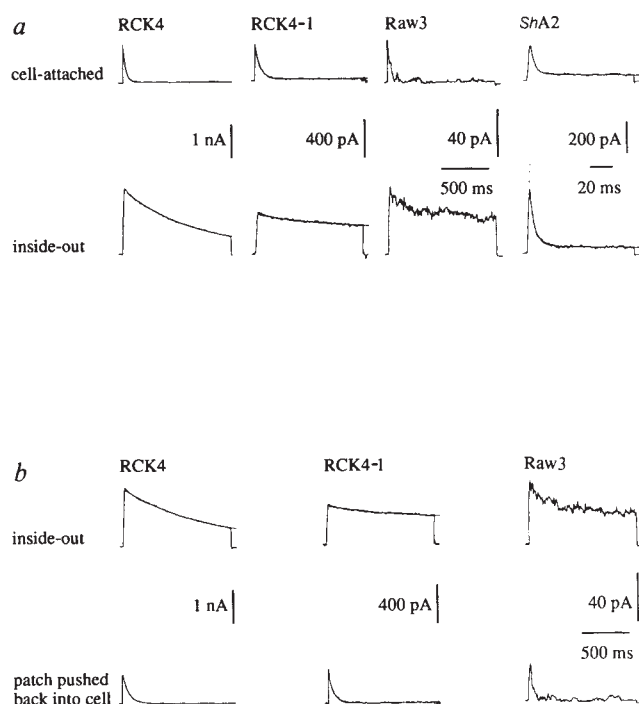


FIG. 2 Comparison of four fast-inactivating K^+ channels (RCK4, RCK4-1, Raw3, *ShA*2) in cell-attached and inside-out patches. Test pulses and recording as described for Fig. 1. **a**, Fast inactivation disappears after excision of the cell-attached patch for RCK4, RCK4-1 and Raw3 channels but not for *ShA*2 channels. **b**, Loss of fast inactivation is reversed after pushing the patch back into an oocyte. Patches with RCK4-1 and Raw3 channels were pushed into the same oocyte from which the patch was obtained. The time constants of the restored fast inactivation became the same as before in the cell-attached configuration. In the case of RCK4 we used an oocyte for patch-clamping different from where the patch was obtained. This resulted in a change in the time constant from 28 ms in the cell-attached configuration to 65 ms after cramming.

METHODS. Excised inside-out patches were kept in K-int solution containing (in mM): KCl (100), $MgCl_2$ (2), EGTA (10), HEPES (10) (to pH 7.2 with KOH (20)). K-int solution was put into the recording chamber after some minutes of vortexing in air to allow equilibration of the oxygen partial pressure.

Surprisingly, the mammalian transient K^+ channels lost their fast component of inactivation after formation of inside-out patches (Fig. 2a, first, second and third columns), whereas the *Drosophila Shaker* K^+ channels *ShA2* (ref. 16) did not (Fig. 2a, fourth column). The loss of inactivation was similar over the whole voltage range and for currents in inward and outward directions measured in symmetrical K^+ solution (results not shown). Fast inactivation could not be restored in the inside-out patches by varying the pH, ionic concentration, or by adding ATP or cyclic AMP to the bath solution facing the cytoplasmic side of the patch (results not shown). Fast inactivation reappeared after pushing (patch cramming)¹⁷ the inside-out patch back through the membrane of an oocyte (Fig. 2b) so that the cytoplasmic side of the patch was again exposed to the cytoplasm of the cell.

As shown in Fig. 3a, fast inactivation could also be fully restored in inside-out patches by application of the reduced form of glutathione (GSH). After 10 s of exposure the time constants in the experiments illustrated in Fig. 3a became even faster than in the cell-attached configuration. Antioxidant substances like dithiothreitol also had a restoring effect (Fig. 3b). This indicates that the effect of GSH is based on its antioxidant action, rather than on some other effect specific to GSH.

The N-terminal end of the *Drosophila Shaker* K^+ channel protein forms a fast-inactivating gate called the 'ball domain'^{18,19}. If such a mechanism also exists in mammalian $I_K(A)$ channels there are two ways in which these channels might lose fast inactivation: as a result of an alteration either in the binding-site for the ball domain or in the ball domain itself. To check whether there was a functional binding site for the 'ball domain', we applied a peptide corresponding to the first 37 amino acids of RCK4 (see legend to Fig. 3) to the cytoplasmic side of a patch with RCK4-1 channels that had lost fast inactivation. As shown in Fig. 3c, this peptide could mimic fast inactivation of the channels (like the analogous peptide derived from *Shaker* K^+ channels¹⁹). This indicates that the loss of fast inactivation is not due to alteration of the binding site for the ball domain, but rather to a change in the ball domain itself.

It is well known that cysteine-containing proteins are prone to oxidation. Comparison of the N-terminal sequences shows that there are two cysteine residues in the ball domain of Raw3, one in the ball domain of RCK4, but none in the corresponding *Shaker* sequence. To investigate whether these cysteines are responsible for loss of fast inactivation, we constructed a mutant RCK4-C13S channel in which the cysteine at position 13 in the ball-domain of RCK4 was replaced with serine. The mutant channels no longer showed altered properties when the patch was excised. Fast inactivation was unchanged under exactly the same conditions in which fast inactivation of the wild-type channels was lost (Fig. 4a). This strongly suggests that the N-terminal inactivation gate of RCK4 requires cysteine 13 in the reduced form for its function.

This should also hold for RCK4-1 channels, which have the same inactivation gate in their RCK4 subunit. In Raw3 channels the first cysteine residue in the N-terminal region is situated in the sequence (SSVCVS), which is homologous to the corresponding RCK4 sequence (SSGCNS). This suggests that cysteine 6 has a similar role in Raw3 channels.

If the interconversion between oxidized and reduced forms of the channels is slow, then a rapid component of inactivation (reduced channels) and a much slower component (oxidized channels) should be seen. But the time course in Fig. 1a (right) cannot be fitted by superimposition of the fast and the slow time courses shown in Fig. 2a (second column). This suggests that interconversion is quite fast and controls the time course in Fig. 1a (right). We therefore measured the speed of disappearance and reappearance of fast inactivation.

The loss of inactivation after excision of the patch occurred in less than 500 ms. The first eight traces in Fig. 4b were measured

in the cell-attached configuration. Intervals between the pulses were only 500 ms, leading to a decrease in current because of inactivation. The patch was excised between pulses eight and nine. Fast inactivation disappeared immediately after excision of the patch whereas the recovery from inactivation had its normal time course (increase of the current during pulses nine to fifteen). Obviously the process of disabling the ball domain is rather fast but only involves those balls that have already recovered from inactivation, namely those not bound to the inner mouth of the channel. The speed of restoring fast inactivation was measured with GSH (5 mM) applied to inside-out patches with a fast-application system. GSH produced a decay

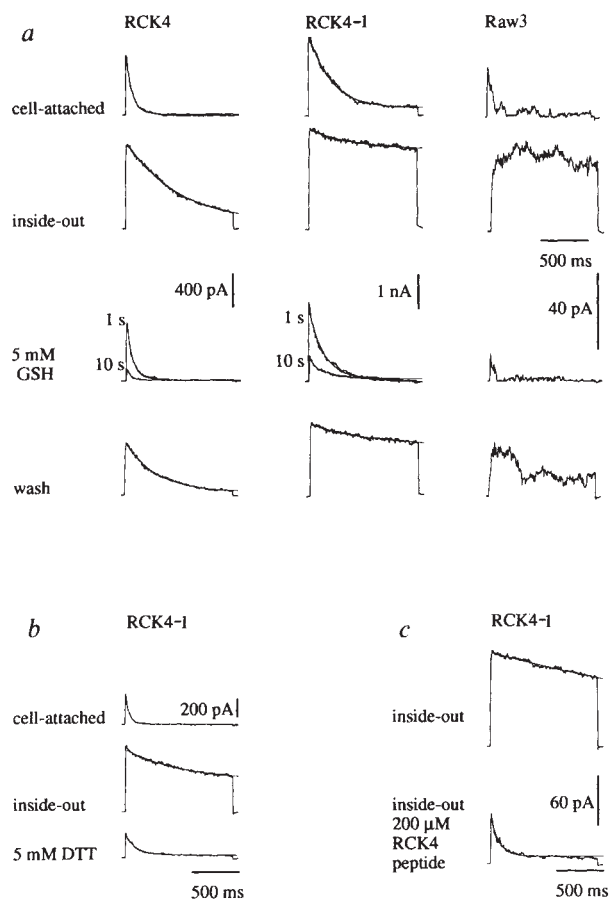
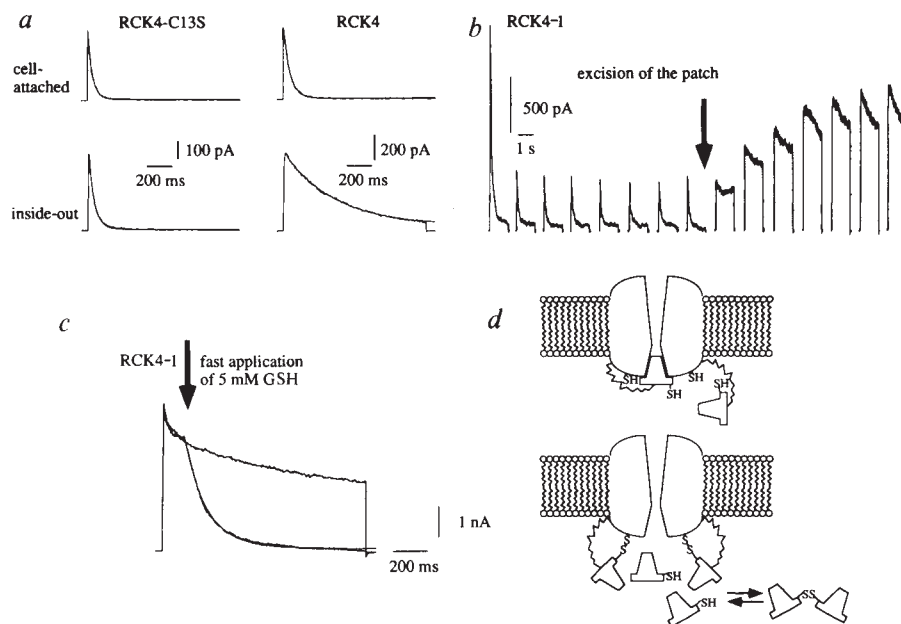


FIG. 3 Loss of fast inactivation is reversed by antioxidant compounds and by the N-terminal ball domain of RCK4. *a*, Time constants of fast inactivation were 64 ms for RCK4 and 234 ms for RCK4-1 in the cell-attached configuration (first row of traces). The loss of fast inactivation (second row of traces) in RCK4, RCK4-1 and Raw3 is reversed by 5 mM GSH after 1 s (64 ms for RCK4 and 154 ms for RCK4-1) and becomes even faster than in the cell-attached configuration (41 ms for RCK4 and 57 ms for RCK4-1) after 10 s (third row of traces). The effect can be washed-out again (fourth row of traces). *b*, Dithiothreitol (DTT; 5 mM) could also restore fast inactivation of RCK4-1 channels. *c*, Inside-out patch with RCK4-1 channels that have completely lost fast inactivation. Application of a peptide corresponding to the first 37 amino-acid residues of the RCK4 sequence in a concentration of 200 μ M to the cytoplasmic side of the patch mimics fast inactivation. METHODS. GSH and dithiothreitol (Sigma) were dissolved in K-Int (see legend to Fig. 2) and applied with an application pipette having a diameter of 30 μ m. The application pipette was moved manually. Therefore the time of 1 s after application given in *a* is approximate. The N-terminal peptide of RCK4 was synthesized in a continuous flow instrument constructed and operated as described²³. Peptide chain assembly was performed on a 1% crosslinked polystyrene support using Fmoc chemistry and *in situ* activation by BOP (ref. 24). Purification was by reversed-phase HPLC. The N-terminal peptide of RCK4 has the sequence MEVAMVSAESSGCNSHMPYGYAAQARARERERL-AHSR (in one-letter code).

FIG. 4 Loss and restoration of fast inactivation of mammalian K^+ channels is a fast process depending on a cysteine residue in the channel protein. **a**, Comparison of the mutant RCK4-C13S with wild-type RCK4 channels in cell-attached and inside-out patches. RCK4-C13S channels do not lose fast inactivation after excision of the patch (left column). Inside-out patches were exposed to K-Int solution (see Fig. 2 legend) at pH 7.2. The time constant of inactivation depends on the pH of the solution facing the cytoplasmic side of the patch (not shown), lying between 9.7 and 11 ms at pH 6, between 45 and 56 ms at pH 7.2, and between 70 and 141 ms at pH 8. RCK4 wild-type channels were used under identical conditions in a subsequent experiment (right family of traces). After excision of the patch RCK4 channels lose their fast inactivation. The remaining slow inactivation of RCK4 was less dependent on pH, having a decay time constant of between 169 and 259 ms at pH 6, between 344 and 627 ms at pH 7.2 and between 318 and 455 ms at pH 8. **b**, Time course of loss of fast inactivation following excision of a cell-attached patch with RCK4-1 channels. Test pulses of 1.2 s went from -80 to 40 mV with 500-ms intervals. In the intervals between the test pulses only 25% of the channels could recover from inactivation. The current in response to the second test pulse is therefore only 25% of that in response to the first. Excision of the patch was made manually between the eighth and the ninth test pulse. The current in response to the ninth test pulse has already completely lost fast inactivation but has the same amplitude as pulse eight. The current increase during the following pulses reflects the normal time course of recovery from fast inactivation, and is also found in cell-attached measurements of RCK1,4 channels¹⁵. **c**, Fast application of GSH to RCK4-1 channels in an inside-out patch. Two consecutive test pulses of 1.2 s were applied with an interval of 20 s. At 120 ms after the onset of the second test, 5 mM GSH was applied with a fast application system. The current decayed to zero with a time constant of 588 ± 138 ms ($n=4$). **d**, Possible model of formation of disulphide bridges between ball domain and the channel protein. In the reduced state the ball can move freely. A disulphide bridge can only form when the ball is not bound to the pore (upper part). In the oxidized state the ball domains are immobilized by the disulphide bridges. The binding site is not altered, so free peptides can still bind to the pore and mimic inactivation. It is possible that parts of the free peptide molecules applied in solution are also inactivated by oxidation.



METHODS. The mutant RCK4-C13S was constructed according to ref. 25. The mutant primer was MC13S (27 nucleotides) 5'-CATGTGGCTGTTGGACCCGTGAGCTCTC-3' (nucleotides +25 to +51, from ref. 9). The flanking primers were the SP6 promoter primer 24 (New England Biolabs) and p4-RCK4 (20 nucleotides) 5'-ATAGAGATTAAGATGACCAG-3' (nucleotides +949 to +968, from ref. 9). The fast application system consists of a double-barrel pipette with a tip diameter of 30–40 μ m and a piezo manipulator moving the patch pipette. One barrel of the application pipette was filled with K-Int, the other one with 5 mM GSH dissolved in K-Int. Driven by the piezo, the recording pipette with the inside-out patch stepped from the stream of solution coming out of one barrel to the other. The exchange time estimated with a patch going from K-Int to K-Int containing 30 mM CsCl was estimated to be less than 3 ms (results not shown).

of the current with a time constant which was of the same order of magnitude as fast inactivation (Fig. 4c). This indicates that the rate of fast inactivation in the mammalian $I_K(A)$ channels might be controlled not only by binding the inactivating ball domain, but also by the slower process of immobilizing and remobilizing the inactivating gate.

In conclusion, the inactivation behaviour of mammalian $I_K(A)$ channels is regulated by intracellular compounds that reduce or oxidize cysteine residues. This regulation is probably based on a fast and reversible formation of disulphide bridges between the inactivating gate, or ball domain, and an as-yet-unknown part of the protein. Apparently, the formation of disulphide bridges occurs only in the closed and open state of the channel when the inactivating gate is open, but not in the

inactivated state of the channel (Fig. 4d). Under certain conditions, reduction of the disulphide bridges becomes the rate-limiting step of fast inactivation that slows down the time course of inactivation. RCK4 and Raw3 were originally isolated from a rat brain complementary DNA library. They are probably subunits of neuronal $I_K(A)$ channels. Heteromultimeric channels like the RCK4-1 channel acquire their ability to be regulated from the RCK4 subunit. If the level of reduced glutathione, for example in synaptic terminals, were to undergo changes, then this mechanism could change the excitability of the neuron or alter the temporal integration of synaptic inputs. Such a mechanism may underlie the change in the inactivation parameters of the $I_K(A)$ current in *Hermisenda crassicornis* that is correlated with associative learning²⁰. □

Received 28 May; accepted 3 July 1991.

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ACKNOWLEDGEMENTS. We thank B. Sakmann, D. Colquhoun, T. Verdoorn, H. Schirmer, P. Stern and K. H. Schröter for discussions and for reading the manuscript and E. Stein for the mutant RCK4-C13S.

Charge movement during Na^+ translocation by native and cloned cardiac $\text{Na}^+/\text{Ca}^{2+}$ exchanger

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$\text{Na}^+/\text{Ca}^{2+}$ EXCHANGE is electrogenic and moves one net positive charge per cycle^{1,2}. Although the cardiac exchanger has a three-to-one $\text{Na}^+/\text{Ca}^{2+}$ stoichiometry³, details of the reaction cycle are not well defined^{2,4-8}. Here we associate Na^+ translocation by the cardiac exchanger with positive charge movement in giant membrane patches from cardiac myocytes^{9,10} and oocytes expressing the cloned cardiac $\text{Na}^+/\text{Ca}^{2+}$ exchanger¹¹. The charge movements are initiated by step increments of the cytoplasmic Na^+ concentration in the absence of Ca^{2+} . Giant patches from control oocytes lack both steady-state $\text{Na}^+/\text{Ca}^{2+}$ exchange current (I_{NaCa}) and Na^+ -induced charge movements. Charge movements indicate about 400 exchangers per μm^2 in guinea-pig sarcolemma. Fully activated I_{NaCa} densities (20–30 $\mu\text{A cm}^{-2}$) indicate maximum turnover rates of 5,000 s^{-1} . As has been predicted for consecutive exchange models⁴⁻⁷, the apparent ion affinities of steady state I_{NaCa} increase as the counterion concentrations are decreased. Consistent with an electroneutral Ca^{2+} translocation, we find that voltage dependence of I_{NaCa} in both directions is lost as Ca^{2+} concentration is decreased. The principal electrogenic step seems to be at the extracellular end of the Na^+ translocation pathway.

The strategy to isolate electrogenic partial reactions of cardiac $\text{Na}^+/\text{Ca}^{2+}$ exchange in giant excised membrane patches presupposed that the exchanger moves Na^+ and Ca^{2+} in separate steps, either or both of which might be electrogenic^{4,5,7}. Exchanger binding sites are expected to reorient between the cytoplasmic side ('E1' states) and the extracellular side ('E2' states) only when loaded with 3 Na^+ or 1 Ca^{2+} , whereby transitional states with 'occluded' ions may exist ('E0' states) (Fig. 1). If a functional homology with the Na^+/K^+ pump exists, the Na^+ loaded carrier could bear one positive charge¹²⁻²⁰ and electrogenicity would occur during Na^+ 'deocclusion' to the extracellular side (E0-nnn to E2-nnn transition) and/or during the actual unbinding of Na^+ from the extracellular side¹²⁻¹⁷ (E2-nnn to E2-o).

Ion-jump experiments were designed to test these possibilities in inside-out excised patches. Low concentrations of either free Ca^{2+} (10 μM in Fig. 2a, b) or Na^+ (5 mM in Fig. 2c) were included in the pipette. Then, in the absence of cytoplasmic Ca^{2+} and Na^+ , only the reactions moving both ions and binding sites from the extracellular side to the cytoplasmic side (E2 to E1) should occur. The exchanger would thus orient uniformly with empty binding sites on the cytoplasmic side (E1-o). A pulse of cytoplasmic Na^+ should then move binding sites to the extracellular side (E2), generating a current transient if a net charge moves through membrane electrical field.

As shown in record 1 of Fig. 2a, rapid application of 40 mM cytoplasmic Na^+ in the presence of 10 μM extracellular $[\text{Ca}^{2+}]$ (no cytoplasmic Ca^{2+}) activates the predicted current transient and a small steady state outward I_{NaCa} . The presteady-state transient is abolished by preapplication of submicromolar cytoplasmic free $[\text{Ca}^{2+}]$ (record 2 of Fig. 2a) with a K_i of 70 nM (Fig. 2b). This fulfills an important prediction that preapplied cytoplasmic Ca^{2+} will move binding sites to the extracellular side and thereby preclude a response to cytoplasmic Na^+ . Also as expected, preapplied cytoplasmic Na^+ inhibited charge move-

ments with Hill coefficients >2 (Fig. 2d). Rapid application of Ca^{2+} caused either a small outward current transient as in record 3 of Fig. 2a or no response. Sham solution changes have no effect (record 4 of Fig. 2a).

Figure 2c shows Na^+ -induced current transients obtained when the extracellular (pipette) solution contains 5 mM Na^+ and no Ca^{2+} (10 mM EGTA). On the basis of both geometric and capacitance estimates of membrane surface area, the transients reflect 250–450 charges moved per μm^2 . The Na^+ -induced current transients are blocked reversibly by exchange inhibitory peptide²¹ (K_i , 11 μM), and by Ni^{2+} (K_i , 1.2 mM) (records 2–4). Charge movements were also abolished (not shown) by cytoplasmic Ba^{2+} (K_i , 50 μM) and dichlorobenzamil ($K_i < 10 \mu\text{M}$). Small inverse current transients on removing cytoplasmic Na^+ in Fig. 2c probably reflect the reverse Na^+ translocation reaction.

Definitive association of Na^+ -induced/ Ca^{2+} -blocked current transients with the $\text{Na}^+/\text{Ca}^{2+}$ exchanger has become possible by the cloning and expression in *Xenopus* oocytes of the cardiac exchanger complementary DNA¹¹. Large Na^+ -induced/ Ca^{2+} -blocked current transients (see records 1 and 2, Fig. 3) were present in giant patches from exchanger-expressing oocytes but were absent in control water-injected oocytes (record 3, Fig. 3). Preapplication of cytoplasmic Ca^{2+} inhibited charge movements with a K_i of 100 nM (Fig. 3b), and the Hill coefficient for inhibition by Na^+ was 2.6 (Figs 3c, d). Also, a jump of cytoplasmic Ca^{2+} from 0 to 5 μM (record 4) resulted in either a small outward current transient or no effect. Charge movement densities were 150–500 μm^{-2} .

Analysis of the steady-state I_{NaCa} (Fig. 4) provides independent evidence that the 'deregulated' exchanger¹⁰ functions in a consecutive cycle with voltage-dependent Na^+ translocation. All results are with only one transported ion species on each membrane side ('zero-trans' conditions) and are fitted to the model

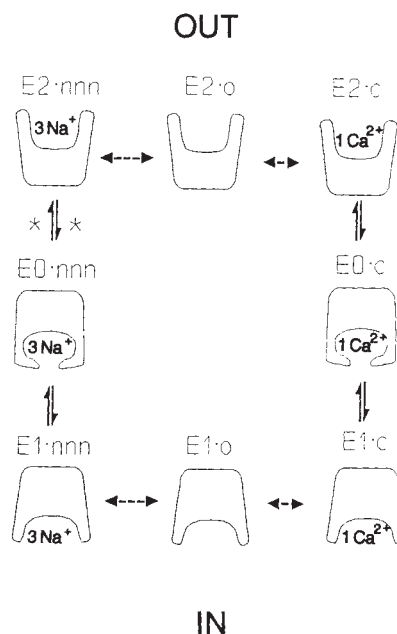
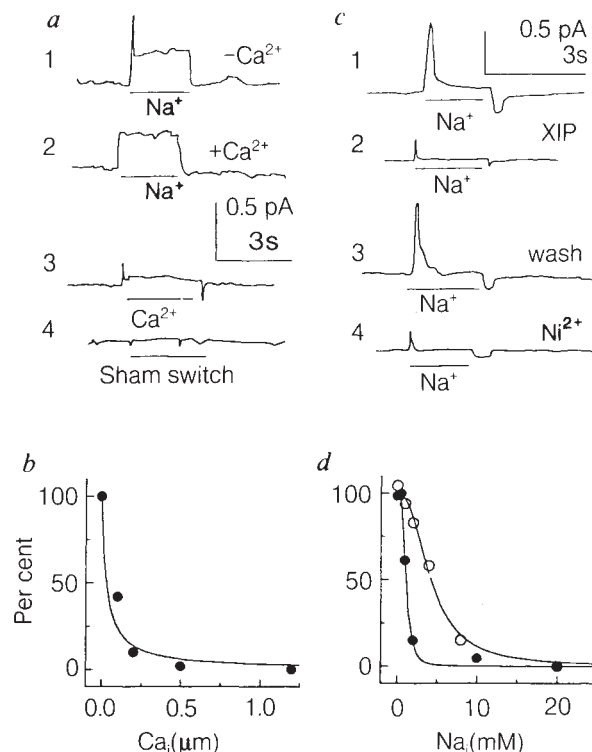


FIG. 1 Hypothetical $\text{Na}^+/\text{Ca}^{2+}$ exchange cycle. States with binding sites oriented to the cytoplasmic side are designated E1 states, those with binding sites oriented to the extracellular side are designated E2 states. Transitional states with occluded ions are designated E0 states. The Na^+ -loaded carrier bears one positive charge. As diagrammed and modelled to reproduce data of Fig. 4, charge movement through the membrane electrical field takes place during the occlusion/deocclusion step (*) at the extracellular side of the Na^+ translocation path (E2-nnn-E0-nnn transitions). Ion-binding reactions (dashed arrows) are treated as instant equilibrium reactions in reproducing the data. (One possible alternative is that charge movement arises from the actual unbinding of one Na^+ from a deep ion well, inaccessible to Ca^{2+} , in the E2 configuration.)

FIG. 2 Na^+ -induced/ Ca^{2+} -blocked charge movements in giant inside-out cardiac membrane patches. *a, b*, No extracellular Na^+ and $10 \mu\text{M}$ free extracellular Ca^{2+} , set with calcium electrode using 10 mM EGTA. Record 1, current transient and small steady state current induced by replacement of 40 mM cytoplasmic Li^+ with Na^+ ; no cytoplasmic Ca^{2+} . Steady-state current is a small outward I_{NaCa} by all criteria used to identify exchange currents⁹ (about 5% of maximum outward I_{NaCa}). Solution switch time is $\sim 100 \text{ ms}$. Time course of current transients was consistently slower in patches that rose up in the pipette tip, indicating that these time courses are probably diffusion-limited. Record 2, same as record 1 in the presence of $0.2 \mu\text{M}$ free cytoplasmic Ca^{2+} . Record 3, small outward current transients induced by application of $5 \mu\text{M}$ free cytoplasmic Ca^{2+} (10 mM EGTA); record 4, switch between same cytoplasmic solutions. *b*, Concentration dependence of block of transient current by cytoplasmic Ca^{2+} . Data plotted as per cent of maximal integrated charge movement in absence of preapplied Ca^{2+} . *c*, Inhibition of Na^+ -induced charge movement by $\text{Na}^+/\text{Ca}^{2+}$ exchange blockers. All records are for replacement of 40 mM cytoplasmic Li^+ with Na^+ . $5 \mu\text{M}$ extracellular Na^+ and no Ca^{2+} . Record 1, control; record 2, $20 \mu\text{M}$ exchange inhibitory peptide (XIP); record 3, after wash-out of XIP. EGTA reduced to $50 \mu\text{M}$; record 4, 5 mM Ni^{2+} with $50 \mu\text{M}$ EGTA. The time course of transients is relatively slow in these records, as the membrane rose up in the pipette tip by $\sim 40 \mu\text{m}$. *d*, Inhibition of Na^+ -induced/ Ca^{2+} -blocked charge movement by preapplication of Na^+ (Hill coefficients, 2.3–3.2 in nine experiments). This relationship reflects the equilibrium of binding sites across the membrane; extracellular Na^+ moves sites inward whereas cytoplasmic Na^+ moves sites outward. In good agreement with theory, half-inhibition with 5 mM extracellular Na^+ (●) occurs at fourfold lower cytoplasmic Na^+ concentration than with 25 mM extracellular Na^+ (○). The fact that half-inhibition occurs at a lower cytoplasmic $[\text{Na}^+]$ than the extracellular $[\text{Na}^+]$ suggests that the Na^+ translocation pathway is asymmetrical. Current transients in the presence of 150 mM extracellular Na^+ (not shown) were either greatly reduced or absent.

METHODS. Guinea-pig myocytes were isolated and giant cardiac membrane patches ($16\text{--}22 \mu\text{m}$ with $2\text{--}10 \text{ G}\Omega$ seals) were formed as described^{9,10}. Cardiac $\text{Na}^+/\text{Ca}^{2+}$ exchange was expressed in oocytes by injection of cRNA as described previously¹¹. Control oocytes were injected with water. The vitellin layer of oocytes was removed mechanically in hypertonic solution, and giant membrane patches were formed and excised with diameters of 25 to $35 \mu\text{m}$ and seal resistances of $2\text{--}10 \text{ G}\Omega$. Charge movement measurements are at 22°C . Steady-state current measurements in Fig. 3 are at 34°C . In all experiments, the exchange was 'deregulated' by treatment with 1 mg ml^{-1} α -chymotrypsin for 1 min because for steady-state I_{NaCa} measurements, it is essential to prevent the activating effect of cytoplasmic Ca^{2+} (ref. 10). This pretreatment routinely increased the magnitudes of $\text{Na}^+/\text{Ca}^{2+}$ exchange charge movements measured in the absence of cytoplasmic Ca^{2+} . Solutions were switched by moving the pipette tip through the interface of two solution streams flowing rapidly from the end of a double-barrelled, theta-style glass pipette. This was done in one of three ways with identical results: the microscope stage could be moved rapidly, the pipette superfusion chamber with mounted theta-pipette could be moved using a solenoid or the solution directed at the patch could be switched by rapid variation of pressure driving solution flow from the individual lines. As estimated from I_{NaCa} changes on stepping cytoplasmic Na^+ to nonsaturating concentrations and on removing Na^+ , 80% solution equilibration at the membrane surface ranged from $0.1\text{--}1.0 \text{ s}$, being slowest in patches that extended into the pipette tip. Records presented are filtered at 20 or 40 Hz . To validate our methods and procedures, partial electrogenic reactions of the Na^+/K^+ pump^{13–19} were first identified under appropriate conditions using Mg-ATP jumps in myocyte membrane. Total charge moved during Na^+ translocation by the cardiac Na^+/K^+ pump^{14,15} was about $2,000 \mu\text{m}^{-2}$ or $4\text{--}6$ times larger than by the $\text{Na}^+/\text{Ca}^{2+}$ exchanger, as measured in this article. In all charge movement results



presented, any participation of the Na^+/K^+ pump is eliminated by including both $50 \mu\text{M}$ vanadate and 50 mM K^+ in the cytoplasmic solutions. Solutions: in mM, pH was set to 7.0 with NMG (*N*-methyl-D-glucamine) or MES (*N*-morpholino-ethanesulphonic acid); for $\text{Na}^+/\text{Ca}^{2+}$ exchange charge movement: extracellular (pipette) solutions, MgCl_2 (5), TEA-MES (40), HEPES (10), EGTA (10), K-MES (20), NMG-MES (100) and Na-MES as indicated in text and legends. For results in Fig. 2a, CaCO_3 was added to give $10 \mu\text{M}$ free Ca^{2+} . Cytoplasmic solutions, TEA-MES (20), Cs-MES (20), HEPES (10), MgCl_2 (1), EGTA (10), K-MES (50), total Na-MES + Li-MES (100), vanadate ($50 \mu\text{M}$) and CaCl_2 to achieve free Ca^{2+} , as given in text and legends; for outward I_{NaCa} : extracellular solutions, MgCl_2 (2), TEA-MES (20), ouabain (0.2) and CaCl_2 as indicated in legends and text. Free Ca^{2+} concentrations of $10 \mu\text{M}$ were buffered with 10 mM EGTA; cytoplasmic solutions, MgCl_2 (1), TEA-MES (20), Cs-MES (20), EGTA (10) with no Ca^{2+} , HEPES (10) and total Na-MES + Cs-MES (100); for inward I_{NaCa} , cytoplasmic solutions, same as for outward I_{NaCa} , but no Na-MES and Ca^{2+} added as CaCl_2 . Free Ca^{2+} concentrations were checked with Ca^{2+} -selective electrodes. Extracellular solutions, MgCl_2 (4) (except Fig. 4e with MgCl_2 (10)), EGTA (10), TEA-MES (20), Cs-MES (20), HEPES (10), total Na-MES + Li-MES (150) and ouabain (0.2).

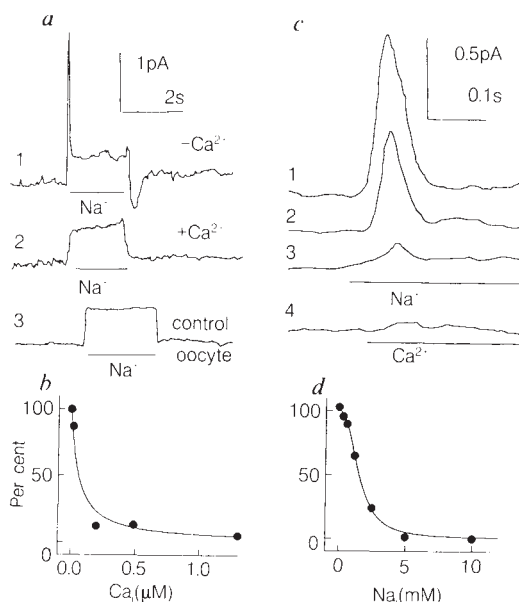


FIG. 3 Na^+ -induced/ Ca^{2+} -blocked charge movement in giant patches from exchanger-expressing oocytes. $5 \mu\text{M}$ extracellular Na^+ and no Ca^{2+} . *a, b*, Record 1, Na^+ -induced charge movement in $25 \mu\text{m}$ oocyte membrane patch. In records 1–3 there is a small increase of steady-state current found in Na^+ -containing solutions at 0 mV both in control and in RNA-injected oocytes. The steady-state current had linear dependence on $[\text{Na}^+]$ up to 100 mM and is assumed to reflect a background Na^+ conductance in the oocyte membrane. Record 2, suppression of Na^+ -induced charge movement by preincubation with $0.2 \mu\text{M}$ free Ca^{2+} . Record 3, typical lack of Na^+ -induced/ Ca^{2+} -blocked charge movement in patch from water-injected oocyte ($31 \mu\text{m}$ electrode tip diameter; no cytoplasmic Ca^{2+}). *b*, Concentration dependence of the block of transient current by cytoplasmic Ca^{2+} . *c, d*, Na^+ -induced current transients at higher time resolution. $32 \mu\text{m}$ patch. Record 1–3, preincubation with 0 mM , 2 mM and 5 mM cytoplasmic Na^+ . Record 4, lack of current transient on application of $5 \mu\text{M}$ free Ca^{2+} ; 10 mM EGTA, no cytoplasmic Na^+ . *d*, Concentration dependence of charge movement inhibition by preapplied cytoplasmic Na^+ .

in Fig. 1 (solid lines). A critical prediction of consecutive transport models is fulfilled in Fig. 4a and b: if Na^+ and Ca^{2+} translocation take place in separate consecutive steps, then the half-maximum concentration (K_d) for one ion species should decrease towards zero as the counterion concentration is decreased towards zero^{4,5}. The K_d for cytoplasmic Ca^{2+} shifts from 4.2 μM at 150 mM extracellular $[\text{Na}^+]$ down to $<1 \mu\text{M}$ at 30 mM extracellular $[\text{Na}^+]$ (Fig. 4a). The K_d for cytoplasmic Na^+ shifts from 17.5 mM at 5 mM extracellular $[\text{Ca}^{2+}]$ to 5 mM with 10 μM extracellular free $[\text{Ca}^{2+}]$ (Fig. 4b). This is similar to results for Ca^{2+} activation of outward I_{NaCa} in myocytes⁶ and Ca^{2+} uptake into reconstituted proteoliposomes containing the cardiac exchanger⁷.

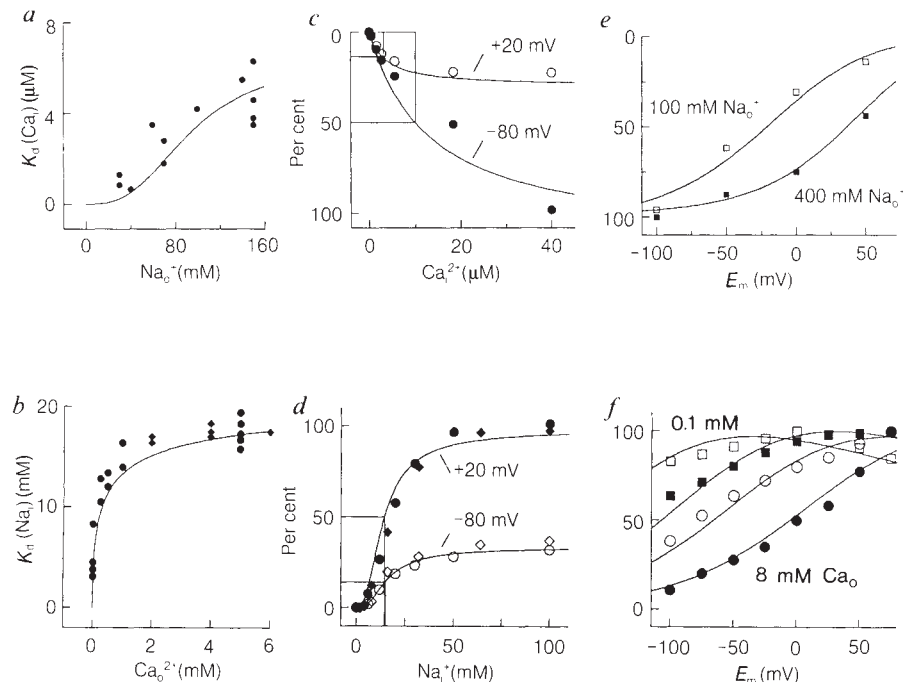
Effects of voltage on cytoplasmic ion dependencies of I_{NaCa} are shown in Fig. 4c and d. When measuring inward I_{NaCa} , the apparent K_d for cytoplasmic Ca^{2+} increases on average $89 \pm 3\%$ (s.e.m.) with stimulation of the current by hyperpolarization from +20 to -80 mV. This reflects a loss of voltage-dependence as the cytoplasmic $[\text{Ca}^{2+}]$ is reduced and Ca^{2+} translocation presumably becomes rate-limiting, consistent with Ca^{2+} translocation being electrically silent. Cytoplasmic $[\text{Na}^+]$ -dependence of outward I_{NaCa} is given in Fig. 4d at -80 and +20 mV. Results from oocyte membrane (diamonds) superimpose with results from cardiac membrane (circles) and demonstrate the equivalence of the cloned exchanger with that in myocyte sarcolemma. There is no shift of the cytoplasmic $[\text{Na}^+]$ -dependence with 100 mV change of membrane potential (Fig. 4d), indicating

that voltage-sensitive steps are not at the cytoplasmic end of the Na^+ translocation pathway. Notably, the cytoplasmic $[\text{Na}^+]$ -dependence of the Na^+/K^+ pump is also insensitive to membrane potential¹⁵.

Extracellular Na^+ strongly affects the current-voltage relation of inward I_{NaCa} (Fig. 4e). With 100 mM extracellular $[\text{Na}^+]$, inward I_{NaCa} doubles with each 50 mV step from +50 to -50 mV, as expected if one charge is moved in a rate-limiting step. With 400 mM extracellular $[\text{Na}^+]$, the current-voltage relation is flatter in this range and saturates with hyperpolarization. The rate of the voltage-dependent reaction apparently increases as extracellular $[\text{Na}^+]$ is increased until a voltage-independent step becomes rate limiting. Similar results are reported for $\text{Na}^+/\text{Ca}^{2+}$, K^+ exchange in the rod outer segment²² and for $\text{Na}^+/\text{Ca}^{2+}$ exchange in squid axon²³.

Like the inward I_{NaCa} , current-voltage relations for the outward I_{NaCa} progressively flatten as the driving $[\text{Ca}^{2+}]$ is reduced, and Ca^{2+} translocation becomes rate-limiting (Fig. 4f). At 0.1 mM (or lower) extracellular $[\text{Ca}^{2+}]$, voltage dependence is nearly lost and a small negative slope develops with depolarization. Both this negative slope and the small outward current transients sometimes found during Ca^{2+} pulse experiments (see, for example, record 3 of Fig. 2a) can be explained by assuming that Ca^{2+} passes through a small fraction of the membrane field (2% in the simulation) on binding and unbinding on the extracellular side. Presumably, binding-site access is more limited in E2 than in E1 states.

FIG. 4 Ion and voltage dependencies of steady-state cardiac I_{NaCa} conform to consecutive exchange model with voltage dependence at extracellular Na^+ release and binding. All solid lines are a least-squares fit of the model in Fig. 1 to a data base including results presented here. a, Dependence of half-maximum cytoplasmic free $[\text{Ca}^{2+}]$ (K_d) for inward I_{NaCa} on extracellular $[\text{Na}^+]$. b, Dependence of half-maximum cytoplasmic $[\text{Na}^+]$ (K_d) for outward I_{NaCa} on extracellular free $[\text{Ca}^{2+}]$. c, Leftward shift of cytoplasmic $[\text{Ca}^{2+}]$ -dependence of inward I_{NaCa} with depolarization; 100% corresponds to the largest inward current magnitude at high $[\text{Ca}^{2+}]$ at -80 mV (20–70 pA). Additional data points at higher $[\text{Ca}^{2+}]$, not shown, were included in data fits. d, Lack of effect of membrane voltage on the cytoplasmic $[\text{Na}^+]$ dependence of outward I_{NaCa} ; 8 mM extracellular $[\text{Ca}^{2+}]$, 100% corresponds to the largest outward current magnitude at high $[\text{Na}^+]$ at +20 mV (30–100 pA). e, Flattening and saturation of inward I_{NaCa} -voltage relationships when extracellular $[\text{Na}^+]$ is increased from 100 to 400 mM. Na-MES replaced by Cs-MES in pipette, and osmolality of all extracellular solutions was equal, 20 μM free cytoplasmic $[\text{Ca}^{2+}]$. I_{NaCa} magnitudes in each current-voltage relation were normalized to values at -100 mV. f, Flattening and saturation of outward I_{NaCa} -voltage relations as extracellular $[\text{Ca}^{2+}]$ is lowered from 8 mM (●) to 2 mM (○) to 0.4 mM (■) to 0.1 mM (□); 30 mM cytoplasmic $[\text{Na}^+]$. I_{NaCa} magnitudes were normalized to largest value occurring in each current-voltage relationship. Note negative slope with strong depolarization with 0.1 mM Ca^{2+} . Rate constants used for data fits to the



model in Fig. 1: E2- nnn -E0- nnn transitions, $10^4/K_{\text{Em}}\text{s}^{-1}$ and $10^4 \cdot K_{\text{Em}}\text{s}^{-1}$, whereby K_{Em} is $e^{0.5 \cdot (1-\tau) \cdot E_m \cdot F/RT}$ and τ (0.02 in the simulation) is the fraction of membrane potential experienced by ions during binding at the extracellular side; E0- nnn -E1- nnn transitions, $1.84 \cdot 10^4\text{s}^{-1}$, E2-c-E0-c transitions, $4.15 \cdot 10^4\text{s}^{-1}$; E0-c-E1-c transitions, $5.17 \cdot 10^4\text{s}^{-1}$. On the cytoplasmic side, the Ca^{2+} dissociation constant (K_d) was 31 μM , and those for sequential Na^+ binding were 32.2, 11.1 and 11.9 mM in the order of first to last ion bound. On the extracellular side, a parameter was introduced to increase the corresponding dissociation constants for Ca^{2+} and the first Na^+ binding reaction (328-fold in the simulations), possibly indicating restricted binding-site access. (For results in e only, this factor was treated as an experiment-specific free parameter to account for a higher extracellular Mg^{2+} concentration (10 mM) used).

As with studies on the Na^+/K^+ pump²⁴, early results on $\text{Na}^+/\text{Ca}^{2+}$ exchange appeared inconsistent with consecutive ion exchange models (see ref. 5 for review). Our work provides strong support for a consecutive $\text{Na}^+/\text{Ca}^{2+}$ exchange mechanism and should facilitate structure-function studies of the cloned $\text{Na}^+/\text{Ca}^{2+}$ exchanger. The results of both ion jump experiments and steady-state I_{NaCa} measurements are inconsistent with Ca^{2+} translocation involving net negative charge movement⁸. We conclude from our site-density estimates and I_{NaCa} measurements that maximum exchanger turnover rates are about $5,000 \text{ s}^{-1}$. As already proposed for the Na^+/K^+ pump¹²⁻¹⁷ and rod outer segment $\text{Na}^+/\text{Ca}^{2+}$, K^+ exchanger^{2,22}, voltage-dependence must reside in the binding and release of extracellular Na^+ or a closely associated occlusion/deocclusion reaction. □

Received 1 February; accepted 14 June 1991.

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ACKNOWLEDGEMENTS. We thank D. C. Gadsby, S. Muallem and A. Collins for discussion, H. Liao for technical assistance, and A. Fabiato and D. C. Gadsby for encouragement. This work was supported by the American Heart Association and the NIH (D.W.H. and K.D.P.). It is dedicated to the memory of Peter Läuger.

Negative and positive selection of antigen-specific cytotoxic T lymphocytes affected by the $\alpha 3$ domain of MHC I molecules

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THE $\alpha 1$ and $\alpha 2$ domains of major histocompatibility complex (MHC) class I molecules function in the binding and presentation of foreign peptides to the T-cell antigen receptor and control both negative and positive selection of the T-cell repertoire¹⁻³. Although the $\alpha 3$ domain of class I is not involved in peptide binding, it does interact with the T-cell accessory molecule, CD8 (refs 4, 5). CD8 is important in the selection of T cells as anti-CD8 antibody injected into perinatal mice interferes with this process⁶. We previously used a hybrid class I molecule with the $\alpha 1/\alpha 2$ domains from L^d and the $\alpha 3$ domain from $Q7^b$ and showed that this molecule

binds an L^d -restricted peptide but does not interact with CD8-dependent cytotoxic T lymphocytes⁷. Expression of this molecule in transgenic mice fails to negatively select a subpopulation of anti- L^d cytotoxic T lymphocytes. In addition, positive selection of virus-specific L^d -restricted cytotoxic T lymphocytes does not occur. We conclude that besides the $\alpha 1/\alpha 2$ domains of class I, the $\alpha 3$ domain plays an important part in both positive and negative selection of antigen-specific cells.

To examine the role of the $\alpha 3$ domain of class I molecules in the responses of alloreactive and antigen-specific cytotoxic T lymphocytes (CTL), we generated two transgenic mouse strains, C3H. L^d and C3H. L^{Q3} , derived from C3H/HeJ ($H-2^k$) mice. C3H. L^d express intact L^d , whereas C3H. L^{Q3} express an L^d molecule whose $\alpha 3$ domain is switched with $Q7^b$. The $Q7^b$ $\alpha 3$ domain differs from L^d at five residues and in addition has a unique three-amino-acid insert at positions 275-277 (ref. 8). We showed that this molecule (L^{Q3}) binds the same viral peptide as L^d but is not recognized by peptide-specific CD8-dependent CTL (ref. 7). Similar observations have been reported with $\alpha 3$ mutant class I molecules not recognized by CD8-dependent CTL, the defect being due to an alteration in the binding site for CD8 (refs 4, 5). Both L^d and L^{Q3} genes contain the same

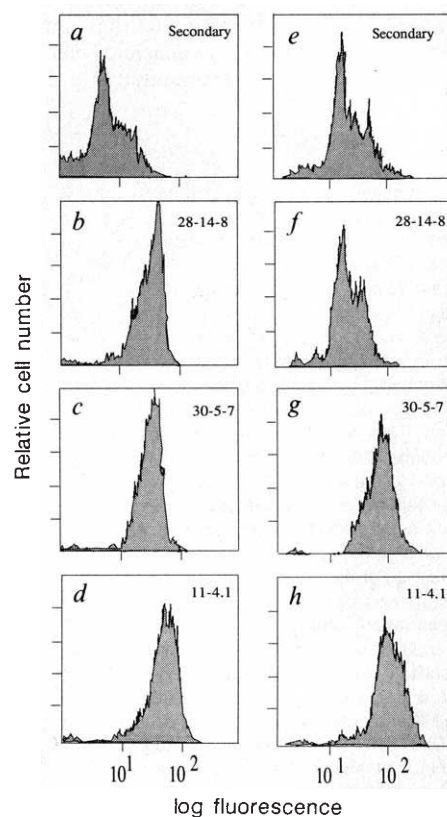


FIG. 1 Cell-surface expression of endogenous or transgene class I expression on spleen cells of C3H. L^d (a-d) and C3H. L^{Q3} (e-h) transgenic mice was confirmed by fluorescent antibody staining and analysis on a FACScan flow cytometer (Becton Dickinson). Antibodies used in these analyses include: a and e, fluorescein isothiocyanate-goat anti-mouse IgG; b and f, 28-14-8 (anti- L^d $\alpha 3$ domain); c and g, 30-5-7 (anti- L^d $\alpha 2$ domain); d and h, 11-4-1 (anti- K^b) (American Type Culture Collection). C3H. L^d and C3H. L^{Q3} express comparable levels of L^d or L^{Q3} , respectively. A similar distribution of antigen is also observed in thymus (data not shown). **METHODS.** C3H. L^d and C3H. L^{Q3} mice were generated by injection of C3H/HeJ oocytes with either a 13.2-kilobase (kb) *PvuII/SalI* fragment containing the 27.5.27 L^d gene⁸, or a 9.9-kb *SphI* fragment of the L^{Q3} hybrid class I gene construct⁷. Each transgene includes the 5' upstream regulators and promoters for wild-type L^d , and expression is detected on lymphoid tissues as expected. Mice carrying the transgene were identified by dot-blot analysis using a pBR322 probe.

L^d promoter, and antigen expression by these transgenic strains is similar (Fig. 1).

We tested the ability of C3H spleen cells to respond to L^d or L^{Q3} . C3H anti-C3H.L L^d primary lymphocyte culture (MLC) effector cells recognize L^d but not L^{Q3} , and this lysis is readily inhibited by anti-CD8, in agreement with our previous findings⁷ (Fig. 2a). When C3H.L $Q3$ spleen cells are used to stimulate a primary *in vitro* MLC, recognition of L^d does occur, and this response is also inhibited by anti-CD8 (Fig. 2b). Although no lysis is detected on L^{Q3} targets, in some experiments a low response is seen to L^{Q3} (data not shown). Thus, spleen cells from L^{Q3} mice do stimulate a primary *in vitro* anti- L^d response. The ability of L^{Q3} transgenic cells to sensitize anti- L^d CTL is probably due to the relatively high levels of this molecule on antigen-presenting dendritic cells⁹. This would be consistent with the data of Alexander *et al.*¹⁰, who show that antigen density affects the extent of CD8 dependency of T-cell recognition. Accordingly, the CTL generated should be biased towards more efficient recognition of L^d versus L^{Q3} on L-cell targets that express relatively low levels of antigen, presumably because the molecule has a wild-type L^d $\alpha 3$ domain.

Secondary C3H anti- L^d recognize L^d , and unlike primary cultures, these cells are relatively resistant to blocking by anti-CD8 (ref. 7) (Fig. 2c). This is a characteristic of many secondary alloreactive CTL^{11,12}. L^{Q3} targets are also recognized by these effectors⁷, but are readily blocked by anti-CD8 (Fig. 2c). This finding is similar to that reported by Salter *et al.*⁵, who noted that T-cell recognition of $\alpha 3$ mutant class I molecules is particularly sensitive to anti-CD8 blocking. Secondary C3H anti- L^{Q3} CTL recognize both L^d and L^{Q3} and these responses are resistant to blocking by anti-CD8 (Fig. 2d). Thus, stimulation with these molecules illuminates a hierarchy of CD8 dependence, presumably reflecting differences in T cell-receptor affinities for antigen. Only relatively high-affinity anti- L^d CTL recognize L^{Q3} .

As our data show that only relatively high-affinity CTL recognize L^{Q3} , we investigated whether expression of the L^{Q3} molecule during T-cell development in C3H.L $Q3$ animals eliminates all L^d -reactive T cells from the repertoire. We therefore cultured L^{Q3} responder cells with L^d stimulators and found that they do respond to L^d , albeit weakly (Fig. 3a). Addition of supernatant containing interleukin-2 (IL-2) to this culture enhanced this response (Fig. 3e). We restimulated these cultures for two further weeks and found strong L^{Q3} anti- L^d CTL activity (Fig. 3c). These CTL are very sensitive to anti-CD8. A dilution of anti-CD8 monoclonal antibody, which fails to inhibit a CD8-dependent

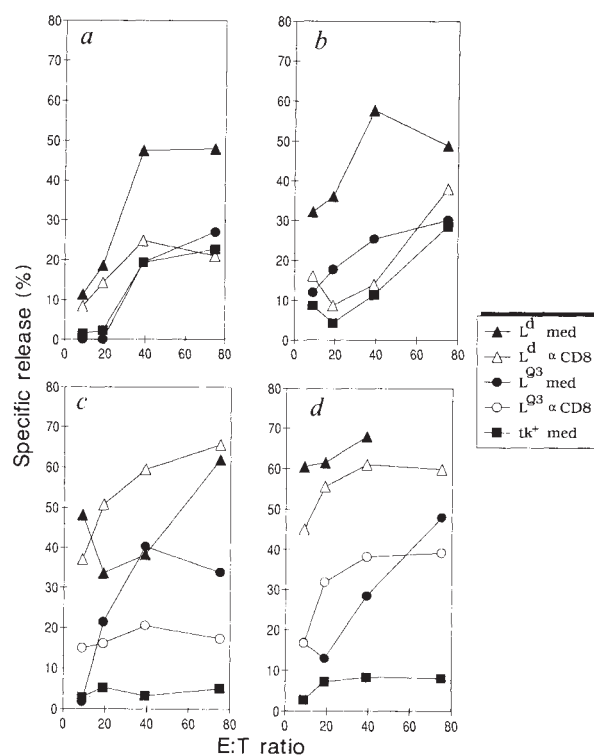
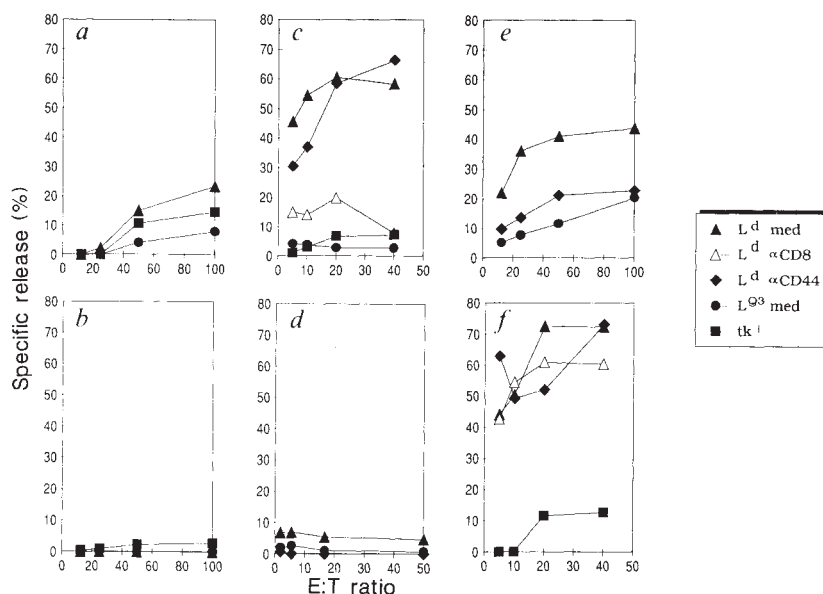


FIG. 2 C3H.L L^d or C3H.L $Q3$ spleen cells stimulate anti- L^d responses. a, C3H anti-C3H.L L^d or b, C3H anti-C3H.L $Q3$ primary *in vitro* MLC, d6 assay. c, Secondary C3H anti-C3H.L L^d or d, C3H anti-C3H.L $Q3$ CTL generated from C3H mice that rejected L^d or L^{Q3} skin grafts (about day 13), were immunized intraperitoneally with 50×10^6 C3H.L L^d or C3H.L $Q3$ spleen cells, respectively, after >3 weeks and restimulated *in vitro* on day 7 or 14 and assayed 6 days later. Targets of a standard 6-h ^{51}Cr release assay are L-cell transfectants that express L^d ($\blacktriangle$), L^{Q3} ($\bullet$), or control (■) untransfected cells (thymidine kinase, tk $^+$), assayed in medium alone or L^d ($\triangle$) or L^{Q3} ($\circ$) in the presence of a 1:20 dilution of culture supernatant of anti-CD8 monoclonal antibody YTS169.4 (YT) (ref. 21). L-cell targets were matched for roughly equivalent expression of transfected class I gene products⁷.

primary anti- L^d response (Fig. 3f), completely blocks L^{Q3} anti- L^d CTL, suggesting that receptors on these cells have a very low affinity for L^d (Fig. 3c). Culture of L^d responder cells with L^{Q3} for six days or for more than three weeks with additional stimulators plus IL-2 does not generate CTL activity, as we

FIG. 3 C3H.L $Q3$ responder cells recognize C3H.L L^d in a CD8-dependent manner. a, C3H.L $Q3$ anti-C3H.L L^d or b, C3H.L L^d anti-C3H.L $Q3$ primary *in vitro* MLC generated in the absence of exogenous IL-2, day-6 assay. c, C3H.L $Q3$ anti-C3H.L L^d or d, C3H.L L^d anti-C3H.L $Q3$ CTL lines generated from primary *in vitro* MLC and fed on days 7, 14, and 21 with C3H.L L^d or C3H.L $Q3$ stimulator cells, respectively, and fresh medium plus IL-2 (ref. 22) and assayed on day 24. e, C3H.L $Q3$ anti-C3H.L L^d primary *in vitro* MLC generated in medium plus IL-2, day-6 assay. f, C3H anti-C3H.L L^d CTL line from primary *in vitro* MLC fed on days 7, 14 and 21 and assayed on day 24. Targets of a standard 6-h ^{51}Cr release assay are L-cell transfectants that express L^d ($\blacktriangle$), L^{Q3} ($\bullet$), or untransfected control (tk $^+$) (■), assayed in medium alone; of L^d in the presence of a 1:40 dilution of anti-CD8 culture supernatant ($\triangle$), or L^d in the presence of a 1:500 dilution of control monoclonal anti-CD44 ($\blacklozenge$), which reacts with a molecule present on CTL, which is not involved in killing²³. Similar results were seen using concanavalin A blast target cells (data not shown).



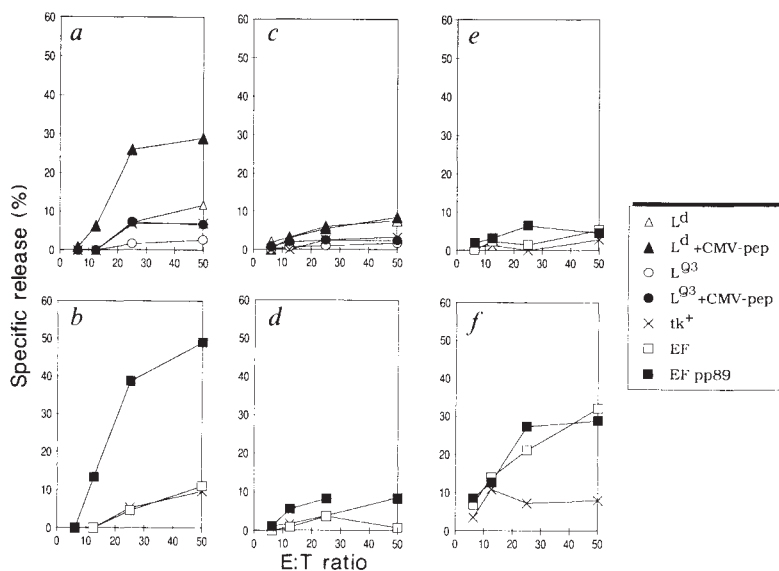


FIG. 4 C3H.L^{Q3} spleen cells do not respond to MCMV nonamer peptide (CMV-pep) presented by C3H.L^{Q3} or C3H.L^d stimulator cells. *a* and *b*, C3H.L^d anti-C3H.L^d+CMV-pep or *c* and *d*, C3H.L^{Q3} anti-C3H.L^{Q3}+CMV-pep or *e* and *f*, C3H.L^{Q3} anti-C3H.L^d+CMV-pep CTL were generated by primary *in vitro* MLC followed by two weekly passages with stimulator cells plus CMV-pep in IL-2-containing medium⁷. In *e* and *f*, data represent two independent experiments. Target cells of a standard 6-h ⁵¹Cr release assay were control L cells (×), or L cells that express L^d (△) or L^{Q3} (○) cultured in the presence (filled) or absence (open) of MCMV peptide (YPHFMPNTL; one-letter amino-acid code) at 500 μg ml⁻¹ for 15 h at 37 °C¹⁴; or SV40-transformed BALB/c embryonic fibroblast cells (EF) untransfected (□) or transfected with the *ie1* gene encoding the MCMV pp89 protein (■)²⁴.

expected (Fig. 3*b, d*). Thus, although expression of the L^{Q3} molecule eliminates high-affinity anti-self T cells, a population of low-affinity L^d-reactive T cells, revealed by their reactivity on L^d but not L^{Q3}, evades this negative selection.

As class I molecules function in positive selection of the antigen-specific repertoire², we next examined the effect of the Q7^b α3 domain on the generation of virus-specific L^d-restricted T-cell responses. We used a synthetic peptide (designated CMV-pep) derived from the immediate early protein pp89 of murine cytomegalovirus (MCMV) which binds to both L^d and L^{Q3} (see legend to Fig. 4)^{7,13,14}. Primary L^d anti-L^d-CMV-pep CTL recognize CMV-pep-pulsed L^d target cells (Fig. 4*a*). This response is blocked by anti-CD8 and these CTL fail to recognize peptide pulsed L^{Q3} targets (Fig. 4*a* and ref. 7). But no anti-CMV-pep activity is detected from L^{Q3} mice (Fig. 4*c*). Further, L^d-CMV-pep, which expresses a native L^d α3 domain and therefore might be recognized by low-affinity L^{Q3}-restricted anti-CMV-pep CTL, is not recognized by L^{Q3} cells (Fig. 4*c*).

We also tested CTL reactivity with target cells that express equivalent levels of L^d, but which differ as to whether they express the MCMV pp89 antigen. L^d anti-L^d-CMV-pep CTL recognize L^d+pp89 cells, but not untransfected cells (Fig. 4*b*), indicating that recognition of L^d-CMV-pep is not due to increased expression of L^d in CMV-pep-treated cells. L^{Q3} anti-L^{Q3}-CMV-pep cultures do not generate anti-L^dpp89 activity illustrating further the inability of these cells to stimulate L^{Q3}-restricted antigen-specific responses effectively (Fig. 4*d*).

As the presence of the L^d α3 domain might allow low-affinity CTL to respond to CMV-pep, we co-cultured L^{Q3} responders with L^d-CMV-pep antigen-presenting cells. These cultures do not generate L^d-restricted CMV-pep-specific CTL (Fig. 4*e, f*). In some experiments we noted L^{Q3} anti-L^d alloreactivity (Fig. 4*f*), as already described (Fig. 3*a, c, e*). But this alloreactivity is reduced compared with cultures in which peptide is omitted (compare Figs 4*f* and 3*c*; also data not shown), presumably because CMV-pep pre-empts self peptides from gaining access to L^d, which contributes to the bulk of alloreactive specificities. This is in agreement with the finding that binding of specific peptides to the L^d molecule influences L^d antigenic structure¹⁵. Regardless of whether an anti-L^d response was detected in these cultures, L^{Q3} anti-L^d-CMV-pep did not elicit detectable antigen-specific responses (Fig. 4*e, f*).

Thus, stimulation of CTL precursors with L^d and L^{Q3} allows us to illustrate a hierarchy of CTL affinities and CD8 interactions *in vitro*, and use of transgenic mice demonstrates that a similar *in vivo* hierarchy of affinities, one of which determines the T-cell

repertoire, is also influenced by the α3 domain of class I. Previous data indicate that the specificity of the T cell receptor plays a major part in determining the T-cell repertoire³. The function of CD8 has been addressed in peripheral T cells where its accessory and co-receptor functions have been demonstrated^{16,17}. CD8 participates in the generation of the repertoire because anti-CD8 antibodies injected into perinatal mice prevent selection of CD8⁺ T cells⁶. Downregulation of CD8 expression has also been noted in transgenic mice bearing anti-self receptors¹⁸. Further, using a mutant mouse strain lacking cell-surface CD8 expression, Fung-Leung *et al.*¹⁹ have shown that CD8 is necessary for the development of functional alloreactive or antigen-specific class I, but not class II, MHC-restricted T cells.

Our data indicate that the α3 domain of class I controls positive selection of the TCR repertoire. This suggests that although CD8-independent CTL with high-affinity receptors for antigen can function in the periphery, their generation is dependent on this co-receptor during ontogeny. This requirement could reflect the action of CD8 as an accessory molecule with low-density class I on thymic stromal cells where education occurs, but we favour CD8 as a co-receptor required for signalling²⁰. The finding that anti-L^d alloreactive T cells are not completely eliminated in L^{Q3} animals could reflect either the retention of low-affinity cells requiring CD8 as an accessory molecule, or a CD8-dependent co-receptor signalling event required for efficient negative selection. □

Received 26 March; accepted 4 July 1991.

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ACKNOWLEDGEMENTS. This work was supported by an NIH grant (J.F.). We thank M. Zuniga for the L^Q gene, G. D. Senn III for technical assistance, and B. Washington for secretarial help.

Co-engagement of CD8 with the T cell receptor is required for negative selection

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ALTHOUGH it is established that the CD8 and CD4 co-receptors are involved in T-lymphocyte recognition and activation in the periphery, it is less clear whether these molecules participate in thymic selection events. Analysis of thymic selection in mice transgenic for T cell-receptor genes^{1–4} or for major histocompatibility complex (MHC) genes⁵, or mice injected with antibodies against CD8, CD4 or MHC molecules^{6–8}, is consistent with the participation of CD8 and CD4 in thymic selection. But antibody-mediated crosslinking of surface receptors in thymic organ cultures⁹ has indicated that CD8 is not involved in thymic deletion. We show here that mice transgenic for a mutant MHC class I molecule that cannot interact with CD8 do not delete CD8-dependent T cells reactive with the wild-type molecule. This finding unequivocally establishes that for negative selection in the thymus, CD8 must interact with the same MHC class I molecule as the T cell receptor.

The role initially proposed for CD8 and CD4 was to bind to MHC class I and class II molecules, respectively, and so increase the avidity of the interaction between effector T cells and antigen-presenting cells^{10,11}. The association of the protein tyrosine kinase p56^{lck} with CD8 and CD4 (refs 12–15) indicates that these coreceptors could also have a function in signal transduction. We have focused on the identification of the binding site for CD8 on MHC class I molecules, and have shown that substitutions at amino-acid residue 227 (refs 16–18) and at other residues between 222 and 229 (ref. 19) of class I molecules prevents this molecule from interacting with CD8. A similar result was obtained using a conjugate binding assay²⁰. We concluded that for effector cell function of CD8-dependent cytotoxic T lymphocytes (CTL), the T cell receptor (TCR) $\alpha\beta$ molecule and CD8 must interact with the same MHC class I molecule. This finding is one of the criteria for distinguishing between the terms co-receptor and accessory molecule²¹.

To establish unequivocally a role for CD8 in thymic selection, we derived mice transgenic for a gene encoding the H-2K^b $\alpha 1$ and $\alpha 2$ domains and the $\alpha 3$ domain from either the wild-type H-2D^d gene (this line is designated 28.1) or a mutant H-2D^d gene (this line is designated 29.7) which has a single amino-acid substitution at residue 227 (Glu $\rightarrow$ Lys) that destroys the interaction with CD8 (ref. 18). The genes used to derive these mice were identical in the 5' and 3' flanking regions to the H-2D^d gene used to demonstrate that transgene-encoded class I molecules function identically to endogenously encoded class I molecules^{22,23}. Our transgenic mice were originally derived on

a (C57BL/6 $\times$ BALB/c) F2 background and were backcrossed to B10.D2 for two generations to obtain H-2^d homozygosity (confirmed by analysis using the polymerase chain reaction (PCR) and oligonucleotides specific for H-2^b or H-2^d class II genes). These mice expressed the product of the transgene on spleen cells at a level slightly above the expression of H-2K^b on spleen cells of H-2^b/H-2^d F1 mice (Fig. 1). Analysis by two-colour fluorescence activated cell sorting (FACS) of class I expression on thymocytes established that cells expressing high levels of H-2^d-encoded class I molecules also expressed a high level of the transgene-encoded class I molecule, and that the ratios of T:B cells and of CD4⁺:CD8⁺ T cells did not alter in the peripheral lymphoid organs of these mice (data not shown). To examine reactivity for H-2K^b in the absence of any other known MHC gene disparity, the transgenic mice were mated with B10.AKM (K^kI^kD^d) and the response to B10.MBR (K^bI^kD^d) was analysed. *In vitro* stimulation of spleen cells from (B10.AKM $\times$ 29.7)F1 mice (transgenic for the mutant H-2K^b gene) with irradiated B10.MBR spleen cells elicited anti-H-2K^b CTL that were cytotoxic for EL4 (H-2^b) cells (Fig. 2a). As these CTL also killed M12.C3 cells transfected with the H-2K^b $\alpha 1\alpha 2$ /H-2D^d wild-type $\alpha 3$ gene (M12.28K^b; Fig. 2b), the determinants recognized by these CTL were present on both wild-type H-2K^b molecules and H-2K^b/H-2D^d exon-shuffled molecules. This CTL population was not cytotoxic for M12.C3 transfectants expressing the H-2K^b molecule in association with the mutated H-2D^d $\alpha 3$ domain (M12.29K^b; Fig. 2c). To confirm that mice transgenic for the gene with the mutant $\alpha 3$ domain (29.7) were not tolerant to H-2K^b $\alpha 1/\alpha 2$ determinants expressed in association with a wild-type $\alpha 3$ domain from either H-2D^d or H-2K^b, spleen cells from the 29.7 mice were stimulated *in vitro* with spleen cells from the 28.1 mice (transgenic for the H-2K^b $\alpha 1\alpha 2$ /H-2D^d $\alpha 3$ wild-type gene). In this strain combination, the only difference in MHC genes is the substitution at residue 227 in the transgene of the responder. CTL generated under these conditions killed both EL4 and the M12.28K^b cells (Fig. 3a and b), establishing that the mutation in the $\alpha 3$ domain of the 29.7 mice resulted in the failure of these mice to delete T cells alloreactive to H-2K^b $\alpha 1/\alpha 2$ determinants expressed in association with a wild-type $\alpha 3$ domain from either H-2D^d or H-2K^b. These CTL were completely inhibited by antibody to CD8 and did not kill the M12.29K^b cells (Fig. 3c), suggesting that there were no autoreactive CD8-independent T cells present in the 29.7 mice. Co-culture of spleen cells from the (B10.AKM $\times$ 28.1)F1 mouse with B10.MBR spleen cells did not generate any

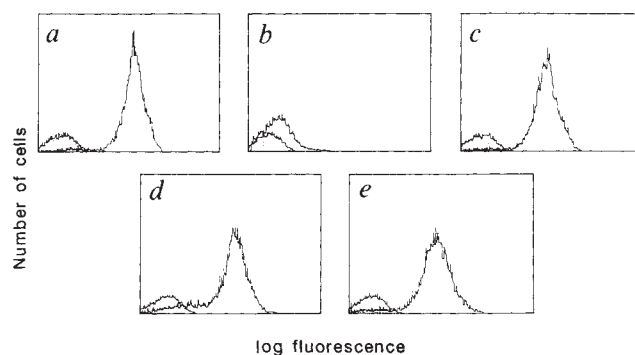
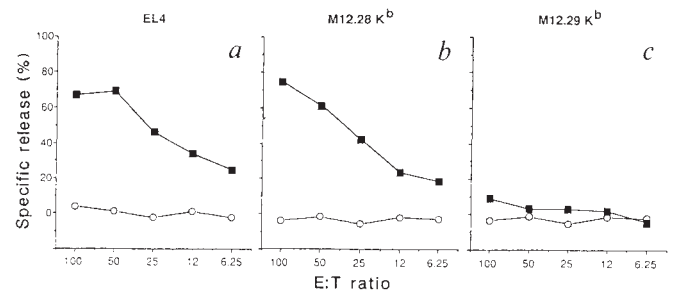


FIG. 1 Flow microfluorometric analysis of H-2K^b expression on spleen cells from a, C57BL/6; b, B10.D2; c, (C57BL/6 $\times$ DBA/2F1); d, e, two of the H-2K^b transgenic lines. The background staining for each population is represented in the same panel.

METHODS. Spleen cells (10^6) were incubated with biotinylated monoclonal antibody EH144 (anti-H-2K^b) for 30 min on ice, washed twice and then incubated for a further 30 min with fluorescein isothiocyanate-conjugated avidin. After staining, cells were washed and the fluorescence of 10,000 viable cells was measured on an EPICS Profile analyser on a logarithmic scale.

FIG. 2 Reactivity of anti-H-2K^b CTL for EL4 and the M12.C3 transfectants. The anti-H-2K^b CTL were generated in a 5-day culture of spleen cells from (B10.AKM $\times$ 29.7)F1 mice (transgenic for the mutant H-2K^b gene) and irradiated B10.MBR spleen cells. The reactivity of this CTL population for a, EL4; b, M12.28K^b (H-2K^b α 1 α 2/H-2D^d α 3 wild type), and c, M12.29K^b (H-2K^b α 1 α 2/H-2D^d α 3 mutant cells), was determined in the absence (■) or presence (○) of antibody to CD8. The level of expression of H-2K^b on the M12.28K^b and M12.29K^b transfectants is identical as determined by flow microfluorimetry (described in Fig. 1 legend; data not shown).

METHODS. The primary CTL were generated by a 5-day culture of 5×10^7 responder spleen cells together with 5×10^7 irradiated (3,000 rad) B10.MBR spleen cells in a final volume of 10 ml DME medium supplemented with 10% FBS. The reactivity of the CTL was assessed by ^{51}Cr release using 10^4 labelled target cells in a final volume of 200 μl . Effector and target cells were incubated together in 96-well V-bottom plates for 5 h at 37 °C. Culture supernatants were collected and specific lysis determined as per cent specific release: $100 \times (\text{experimental release} - \text{spontaneous release}) / (\text{maximum release} - \text{spontaneous release})$. The experimental



release represents ^{51}Cr release from target cells incubated with effector cells either in the presence or absence of a 1:200 dilution of 53-6.72 antibody to CD8; spontaneous release is that obtained in the absence of effector cells and the maximum release is in the presence of 1% Triton X-100.

appreciable CTL activity (Fig. 3d-f), suggesting that substitution of the α 3 domain of H-2K^b with the wild-type H-2D^d α 3 domain had little or no effect on H-2K^b allodeterminants.

Our data establish that mice transgenic for a class I molecule that cannot interact with CD8 are not tolerant to the same molecule possessing a wild-type α 3 domain and that appropriate stimulation *in vitro* can generate CD8-dependent CTL. We interpret this finding as evidence that engagement of the TCR alone is insufficient for negative selection in the thymus and that involvement of CD8 is required. Furthermore, for negative selection to occur, CD8 must engage the same class I molecule as the TCR. This is apparent because negative selection of anti-H-2K^b reactive CTL did not occur, even though thymocytes

of these F1 mice expressed other class I molecules (such as H-2K^d, D^d, L^d, K^k and D^q) that can interact with CD8. One unsolved dilemma concerning thymic selection events is how engagement of the TCR by physiological ligand at different stages of thymocyte differentiation can result in signalling events that induce both positive and negative selection. It is possible that the nature of the signal produced by engagement of the TCR, or alternatively the cellular response to this signal, changes during differentiation. Our finding that co-engagement of the TCR and CD8 by the same molecule is necessary for negative selection suggests a potential signalling role for CD8, possibly mediated by p56^{lck}, in signal transduction during thymic selection. □

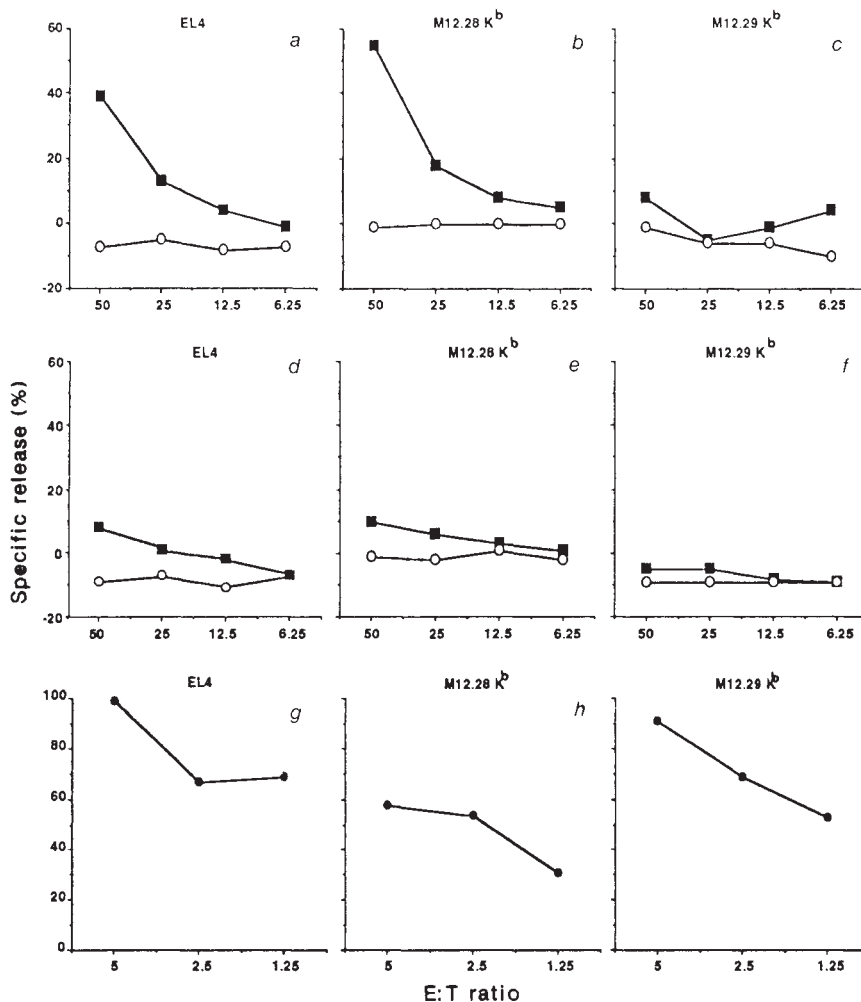


FIG. 3 Reactivity of anti-H-2K^b CTL for EL4 and the M12.C3 transfectants. The anti-H-2K^b CTL represented in a-c were generated in a 5-day culture of spleen cells from (B10.AKM $\times$ 29.7)F1 mice (transgenic for the mutant H-2K^b gene) and irradiated spleen cells from (B10.AKM $\times$ 28.1)F1 mice (transgenic for the wild-type H-2K^b/D^d gene). The CTL in d-f arise from co-culture of (B10.AKM $\times$ 28.1)F1 splenic responder cells with irradiated B10.MBR spleen cells. The CTL in g-i are from a CD8-independent anti-H-2K^b clone, which is used here to demonstrate that M12.29K^b cells are in fact susceptible to lysis by CTL. The reactivity of the first two CTL populations for EL4 (a and d), M12.28K^b (b and e) and M12.29K^b cells (c and f) was determined in the absence (■) or presence (○) of antibody to CD8 (53-6.72). Methodology is described in the Fig. 2 legend.

Received 4 April; accepted 4 July 1991.

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ACKNOWLEDGEMENTS. This work was supported by the National Institutes of Health and the Leila Y. Mathers Foundation. C.L. is a Neurofibromatosis Foundation Young Investigator. T.A.P. is a scholar of the Leukemia Society of America. We thank J. Bernard and J. Zhao for technical assistance.

High risk of squamous cell carcinoma of the cervix for women with HLA-DQw3

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MANY immune responses are controlled by genes of the major histocompatibility complex (MHC)¹. In man these include the loci encoding the HLA-A, -B, -C, -DR, -DQ and -DP antigens, and many diseases have been linked with these^{2–5}. But attempts to identify HLA genes in man that might explain why an immune response against malignant tumours should be ineffective have so far been disappointing, apart from the association reported between the HLA-DR1 antigen and a susceptibility to a rare carcinoma of the thyroid gland⁶. Here we describe another strong connection between a common malignant tumour and an HLA antigen, namely between HLA-DQw3 and squamous cell carcinoma of the cervix: from the 1988 United States tumour registry, 1 in every 63 newborn girls will develop this invasive cancer⁷. We found that 88% of 66 patients had the leukocyte antigen HLA-DQw3 when it would normally be expected in only 50% of individuals. In animals the immune system and the MHC act in defence against virally induced tumours, but until now there has been no evidence that they do so in humans⁸: as squamous cell carcinoma is probably virally induced, our discovery of its association with an HLA antigen will be important to the understanding of the immunogenetic basis of a susceptibility to this tumour.

Blood samples were collected over a 3-year period from 94 patients. The full HLA phenotypes with relevant patient data are given in Table 1. The frequencies of the HLA class II phenotypes of 66 patients with squamous cell carcinoma are compared with the HLA class II phenotypic frequencies of two random caucasian control groups (Table 2). One of these control groups is a local panel consisting of 109 individuals, and the other control group is a caucasian panel of 2,019 individuals from the Ninth International Histocompatibility Workshop⁹. A comparison of the prevalence of HLA-DQw3 antigens in 66 female patients with squamous cell carcinoma and 28 patients with other gynaecological diseases is shown in Table 3. The

frequency of the HLA-DQw3 antigen in the local panel of 109 individuals was found to be 50.4% (International Histocompatibility Workshop panel, 41.2%), whereas the frequency of HLA-DQw3 in the patient group with squamous cell carcinoma of the cervix was 87.8%. This means that a caucasian female with the HLA-DQw3 antigen has a 7.1 times greater chance of developing squamous cell carcinoma compared with caucasian females without this antigen ($P = 0.0009$).

HLA-DQw3 was first defined with alloantisera and is encoded by a locus distinct from that encoding the HLA-DR antigens¹⁰. Two α - and two β -chain genes are present in the *DQ* region of the haploid genome and one $\alpha\beta$ pair encodes the HLA-DQ molecules¹¹. Three different alleles, *DQB1**0301, *DQB1**0302 and *DQB1**0303, code for the three DQw3 antigen subtypes HLA-DQw7, HLA-DQw8 and HLA-DQw9 (refs 12 and 13), but these all share common epitopes which define the broad DQw3 antigen. No preferential association was found with any of the DQw3 subtypes (Table 2). This is not surprising in view of the well-known connection between the HLA-B27 antigen and ankylosing spondylitis which is also an association with epitopes shared by six HLA-B27 allelic products, *B**2701–*B**2706 (refs 2, 3, 14 and 15). All eight HLA-DQw3-negative patients with squamous cell carcinoma of the cervix were positive for HLA-DQw1, but the small sample size prevents speculation about its role in increasing susceptibility.

In our patient group the frequency of the HLA-DR5 antigen was also significantly increased (patients, 54.5%; local random panel, 26.3%; $\chi^2 = 13.74$, $P_{\text{corr.}} = 0.009$). This association is weaker than that between squamous cell carcinoma and HLA-DQw3 and is probably due to linkage disequilibrium between DR5 and DQw3 alleles. We also found significant decrease in the frequency of the HLA-DR6 antigen (17 expected, 2 observed), so females with HLA-DR6 have a 12.7-fold decreased relative risk of developing squamous cell carcinoma of the cervix.

This association between an HLA antigen and susceptibility to a malignant tumour of supposed viral origin is highly significant. The correlation with the HLA-DR1 antigen found for 80% of thyroid cancer patients is comparably strong⁶, but the causative agent of this cancer is still unclear. At least one viral agent has been implicated in the pathogenesis of cervical cancer: human papilloma virus type 16 (HPV-16) has been detected by *in situ* hybridization in the tumours of 90% of patients with squamous cell carcinoma of the cervix but in only 40% of healthy individuals¹⁶. Because many women infected with HPV-16 do not develop cervical cancer, a multiple-hit model involving the interaction of herpes simplex virus (HSV) and HPV has been proposed to explain the development of this cancer¹⁷. An inverse relation between the incidence of HPV and cervical cancer has been found in a population study involving 1,274 women in Greenland and Denmark¹⁸. In Greenland the incidence of cervical cancer among women between 20 and 39 years old is 5.7 times higher than in Denmark, yet the age-adjusted HPV-16/18 infection rate in Greenland is only 67% of that in Denmark. On the other hand, a significantly higher proportion of the Greenland women had antibodies against HSV-2 (68.2%) compared with the Danish women (30.9%).

Interestingly, there is a strong association of HLA-DQw3 with the recurrent form of erythema multiforme¹⁹: this is an episodic, acute, inflammatory disorder of the skin and mucous membranes induced by HSV. All patients with this recurrent form of EM were positive for HLA-DQw3, whereas there were no deviations from the normal HLA allelic frequencies in patients having forms of erythema multiforme not associated with HSV¹⁹. The immune response to HSV may therefore be different in HLA-DQw3 and non-HLA-DQw3 individuals. A small but non-significant association between squamous cell carcinoma and the class I antigen HLA-B12 has been found²⁰; this might be attributable to an immune response allele belonging to the HLA class II family, which is in weak linkage disequilibrium with

TABLE 1 Diagnosis and HLA-A, -B, -C, -DR and -DQ types of patients

Patient	Age	Diagnosis	HLA-A	-B	-Cw	-DR	-DRw	-DQ
K.H.	61	Cervix ca.	3, 24	14, 44	5	1, 5(11)	52, -	1, 3(7)
B.M.	45	Cervix ca.	26, 32	49, -	7, -	1, 5	53, -	1, 3(7)
G.M.	55	Cervix ca.	24, 28	13, 44	5, 6	7, -	53, -	2, 3(8)
P.M.	54	Cervix ca.	2, 33	17, 51	1, 3	3, 4	53, -	2, 3(8)
B.M.	82	Cervix ca.	2, 28	7, 13	6, 7	7, -	53, -	2, 3(8)
P.L.	59	Cervix ca.	1, 24	18, 35	4, -	2, 5	52, -	1, 3(7)
M.M.	44	Cervix ca.	25, 31	13, 39	6, -	7, 5(11)	52, -	2, 3(7)
L.G.	40	Cervix ca.	3, -	27, 44	2, -	5, 8	52, 53	-, 3(7)
K.E.	44	Uterus sa.	1, 2	44, 52	5, -	2, 7	53, -	1, 2
B.E.	53	Uterus my.	11, -	8, 44	7, -	3, 7	52, 53	2, -
L.E.	78	Vulva ca.	3, 24	15, 17	4, 6	7, -	53, -	1, 2
K.C.	50	Cervix ca.	2, 11	35, 44	5, -	4, -	53, -	3, -
G.E.	53	Cervix ca.	28, -	21, -	4, -	5, -	-, -	3, -
H.H.	50	Cervix ca.	2, 11	18, 55	3, 5	5, -	52, -	-, 3(7)
K.U.	54	Uterus my.	2, -	44, 39	5, -	4, 8	53, -	1, 3(8)
N.A.	77	Cervix ca.	3, -	13, 40	3, -	1, 7	53, -	1, 2
P.C.	46	Cervix ca.	2, -	27, -	1, 4	11, 10	52, -	1, 3(7)
H.M.	46	Cervix ca.	2, 24	39, 57	5, -	1, 2	-, -	1, -
L.I.	46	Cervix ca.	2, -	38, 51	1, -	5, -	52, -	1, 3(7)
L.K.	37	Cervix ca.	1, 2	7, 37	5, 7	2, 5	52, -	1, 3(7)
E.A.	34	Cervix ca.	11, 29	44, -	4, -	7, -	53, -	2, 3(7)
M.R.	35	Cervix ca.	2, -	27, 40	3, -	2, 4	52, 53	1, 3(7)
K.R.	54	Cervix ca.	3, 23	44, -	4, -	4, 7	53, -	2, 3(8)
L.S.	60	Cervix ca.	3, 24	27, 44	1, 4	1, 5(11)	52, -	1, 3(7)
H.M.	67	Cervix ca.	2, -	17, 44	5, 6	4, 5(11)	52, 53	-, 3(7)
K.M.	78	Cervix ca.	3, 11	40, 44	5, 6	2, 3	52, -	1, 2
S.A.	49	Cervix ca.	2, 3	21, -	4, -	4, 5	52, 53	-, 3(7)
H.Y.	61	Cervix ca.	1, -	8, 44	5, 7	3, 8	52, -	2, 3(7)
D.T.	84	Cervix ca.	1, 24	7, 18	7, -	2, 5(11)	52, 53	1, 3
W.H.	49	Cervix ca.	2, 11	14, 40	3, -	1, 7	53, -	1, 3(9)
K.A.	49	Cervix ca.	2, -	44, -	4, -	1, 5(11)	52, -	1, 3
K.G.	69	Cervix ca.	2, 29	44, -	2, 5	2, 5(11)	52, 53	1, 3(7)
J.A.	48	Cervix ca.	3, -	51, -	1, -	1, 5(11)	52, -	1, 3
K.B.	28	Cervix ca.	2, 24	35, -	4, 6	7, 5(11)	52, 53	2, 3(7)
B.B.	45	Cervix ca.	1, 2	57, -	6, -	7, 8(6?)	53, -	1, 3
B.M.	33	Cervix ca.	2, 28	14, 44	5, -	5, 8	52, -	3, -
S.R.	32	Cervix ca.	1, -	7, 8	7, -	2, 3	52, 53	1, 2
W.H.	49	Cervix ca.	2, 11	14, 44	5, -	1, 7	-, -	1, 3(9)
B.M.	45	Cervix ca.	26, 32	14, 49	7, -	1, 5	52, -	1, 3(7)
G.M.	48	Cervix ca.	3, 24	14, 49	-, -	1, 5(11)	52, -	1, 3
M.H.	63	Cervicitis	2, 24	41, -	7, -	7, -	-, -	1, 2
S.M.	46	Cervix ca.	2, 32	51, 40	2, -	1, 5(11)	52, -	1, 3(7)
W.M.	83	Cervicitis	2, -	7, 16	5, 7	2, -	-, -	1, -
E.M.	77	Cervix ca.	2, 24	51, -	6, -	2, 4	53, -	1, 3(8)
H.R.	54	Cervix ca.	25, 11	44, 51	4, -	2, 4	53, -	1, 3(7)
S.R.	55	Cervix ca.	1, 2	17, 51	3, 4	4, 5(11)	52, 53	3, -
R.B.	44	Cer. dyspl.	11, 24	38, 39	3, 7	3, 5(11)	52, -	1, 2
A.O.	56	Corpus ca.	11, 24	40, 62	3, -	6(13), 8	52, -	1, 4
K.C.	78	Cervix ca.	3, 28	45, -	4, 7	2, 5(11)	52, -	1, 3(7)
M.M.	48	Mamma. ca.	3, -	18, -	7, -	2(16), -	-, -	1, -
G.G.	36	Cer. dyspl.	1, 3	62, -	3, 7	2, 4	53, -	1, 3(8)
F.M.	29	Cer. dyspl.	24, -	38, 44	1, 4	4, 6(13)	52, 53	1, 3(7)
M.E.	63	Vulvar ca.	2, -	13, 17	6, 7	2, 7	53, -	1, 2
G.K.	48	Cer. polyp	11, 31	5, 17	6, 7	1, 7	53, -	1, 2
K.S.	43	Cer. dyspl.	24, 32	18, 27	2, -	2, 7	53, -	1, 2
K.A.	58	Cervix ca.	1, 31	13, 57	6, -	5(11), 7	52, 53	2, 3
P.R.	84	Cervix ca.	1, 2	6, 2	3, 4	2, 6(13)	52, -	2, 3
W.M.	54	Ad. ca. cer.	3, 23	7, 35	4, 6	1, 4	53, -	1, 3(8)
W.H.	49	Menopause	2, 3	40, -	2, 4	5, 9	-, -	3, -
S.G.	29	Adnexitis	2, 28	51, 50	4, 5	2, 8	52, -	1, 4
G.B.	34	Cer. dyspl.	1, 32	8, 44	7, -	2, 4	53, -	1, 3
L.C.	42	Cer. dyspl.	2, 3	44, -	4, 5	1, 6(13)	52, -	1, -
K.R.	46	Ca. <i>in situ</i>	1, 2	-, -	6, 7	7, 8	52, 53	2, 3
K.P.	53	Cer. dyspl.	2, 26	35, 44	4, 5	4, 5	52, 53	1, 3
S.M.	48	Cervix ca.	2, 11	51, -	4, -	5, -	52, -	1, 3
M.V.	40	Cervix ca.	2, 3	51, -	6, 7	7, 9	53, -	2, 3(9)
W.E.	75	Cervix ca.	11, 32	8, 44	7, -	3, -	52, -	1, 2
G.B.	71	Cervix ca.	-, -	55, -	1, 7	5, -	52, -	2, 3
R.T.	52	Cervix ca.	3, 24	7, 44	7, -	2, 5	52, -	2, 3
J.V.	51	Cervix ca.	2, 25	44, -	1, 7	3, 4	52, 53	2, 3
R.E.	68	Cervix ca.	2, 23	13, 27	1, 4	2, 7	53, -	1, -
S.T.	43	Cervix ca.	1, 2	7, 57	6, -	3, 5	52, 53	3, -
S.Z.	38	Cer. dyspl.	-, -	13, 62	3, -	4, 5(11)	52, 53	3, -
S.A.	57	Cervix ca.	9, 32	45, -	-, -	2, 4	53, -	1, 3
H.E.	45	Cervix ca.	2, 11	15, -	4, -	4, 5	53, -	1, 3
E.S.	59	Cervix ca.	24, -	13, -	4, -	4, 5(11)	52, 53	?, 3(7)
D.K.	39	Cer. dyspl.	2, -	35, 44	4, 5	5, 8	52, -	1, 3
O.K.	35	Cervix ca.	2, 26	7, 38	7, -	4, 9	52, 53	1, 3(7)
B.C.	47	Cervix ca.	3, 28	7, 14	-, -	1, 5	-, -	1, 3
A.M.	62	Cervix ca.	2, 31	27, 44	4, 5	2, 6(13)	-, -	1, -
M.R.M.	38	Cervix ca.	3, 24	7, 44	4, -	5, 9	52, 53	1, 3
J.H.	41	Cervix ca.	2, -	15, -	-, -	5, -	52, -	1, 3
K.I.	64	Cervix ca.	1, 2	8, 40	7, -	2, 5(11)	52, -	1, 3
S.E.	51	Cervix ca.	1, 2	7, 57	6, -	7, -	53, -	1, 3
S.I.	55	Mamma. ca.	1, -	8, 44	5, -	3, 5(11)	52, 53	2, 3
A.E.	45	Cer. dyspl.	26, 28	40, -	3, 7	5, -	52, -	1, -
V.O.	31	Cervix ca.	2, 28	27, 40	3, 5	5(12), 9	52, 53	3, -
L.C.	71	Cervix ca.	2, 28	27, 35	4, -	?, 5(11)	52, -	3, -
A.E.	50	Cer. dyspl.	1, 2	27, -	4, 5	4, 10	52, 53	1, 3
W.D.R.	49	Vulva ca.	2, 32	14, 62	3, -	4, 5(11)	52, 53	3, -
L.E.	53	Cervix ca.	1, 2	7, 13	7, -	3, 5(11)	52, 53	2, 3
W.J.	34	Cervix ca.	3, 26	37, 39	6, 7	2, -	-, -	1, -
G.S.	44	Ad. ca. cer.	24, 43	44, 60	3, -	4, 7	53, -	2, 3
L.E.	58	Ca. <i>in situ</i>	2, 29	7, 18	-, -	1, 5(11)	52, -	1, 3

Peripheral blood mononuclear cells were separated from heparinized blood by density gradient centrifugation²⁴ and used directly for HLA class I (HLA-A, B, C) typing; HLA class II (HLA-DR, DQ) typing was done using B lymphocytes separated by passage over nylon wool columns²⁵. Local antisera supplemented by commercial antisera and sera obtained by exchange comprised the set of 180 HLA class I-specific and 120 HLA class II-specific sera used in this analysis. In addition, the class II trays contained a cytotoxic monoclonal antibody reacting with a DQw3 epitope common to HLA-DQw7, -DQw8 and -DQw9 (RW, unpublished results), the monoclonal antibody R1 (ref. 26), which like the antibody IIB3 reacts with HLA-DQw8 and -DQw9 (ref. 27), and the monoclonal antibody TA10 (ref. 28), which recognizes an epitope of the HLA-DQw7 antigen²⁹. If enough cells were available they were tested with more than 40 local monoclonal antibodies specific for HLA antigens³⁰, as well as with the serum set of the Tenth International Histocompatibility Workshop³¹. Abbreviations: Cervix ca. squamous cell carcinoma of the cervix; Uterus sa., sarcoma of the corpus uteri; Uterus my., myoma of the corpus uteri; Cervicitis, nonspecific inflammation of the cervix; Cer. dyspl., cervical intraepithelial neoplasia; Ca. *in situ*, carcinoma *in situ*, severe dysplasia of the cervical epithelium; Mamma. ca. carcinoma of the breast; Vulvar ca., Vulvar carcinoma; Cer. polyp., cervical polyp; Ad. ca. cer., adenocarcinoma of the cervix.

TABLE 2 HLA class II phenotype frequencies (%) of squamous cell carcinoma patients, local caucasian (LC) and international workshop caucasian controls (IWC)

HLA-	IWC (N=2,019)	LC (N=109)	Patients (N=66)	χ^2	Corrected P value
DR1	18.1	22.0	19.6		
DR2	29.1	22.0	27.2		
DR3	22.6	16.5	10.6		
DR4	23.8	22.9	22.7		
DR5	26.6	26.6	54.5	13.74	0.009
DR6	21.1	28.4	3.0	15.72	0.0036
DR7	22.6	18.3	18.3		
DR8	5.9	9.1	4.5		
DR9	1.6	2.7	6.0		
DR10	1.6	2.7	3.0		
DRw52	ND	70.6	65.7		
DRw53	ND	40.3	56.0		
DQw1	54.2	66.9	59.0		
DQw2	32.9	25.6	27.2		
DQw3	41.2	50.4	87.8	23.5	0.0009
DQw4	ND	5.5	1.5		
		(N=)*	(N=)		
DQw3(7)	ND	(27)	(22)		
DQw3(8)	ND	(8)	(6)		
DQw3(9)	ND	(1)	(3)		

The IWC antigen frequencies used are from the Ninth International Histocompatibility Workshop¹⁰. The local panel (LC) comprises normal controls. Only significant χ^2 values are listed. ND, not determined: either the frequencies of these antigens (w52/w53) or the antigens themselves (HLA-DQw4 and the subtypes of HLA-DQw3, namely 7, 8, 9) were not defined.

* The HLA-DQw3 subtypes of the LC panel and the patients are given as numbers rather than as percentages.

TABLE 3 HLA-DQw3 frequency in 66 patients with squamous cell carcinoma of the cervix and 26 other gynaecological patients

Patient number	Diagnosis	HLA-DQw3 positive	χ^2	P value	Corrected P value
66	Cervix carcinoma	58	23.55	0.0000	0.0009
28	Other gynaecological diseases	14	0.00	NS	NS

The clinical and histological diagnoses of the patients were only disclosed after completion of the HLA typing. We obtained biopsies from 8 patients who were HLA-DQw3-positive and could detect HPV-16 by *in situ* hybridization in all samples (data not shown). The HLA-DQw3 frequencies of both patient groups were compared with the DQw3 frequency obtained in the local panel. The probability (*P*) of the χ^2 value was corrected⁵ for the number of HLA-DQ antigens investigated (*N*=9) using the Bonferroni inequality method as suggested by Dunn³². The relative risk was calculated using Haldane's modification of Woolf's method³³. NS, Not significant.

HLA-B12. In our patient group only the frequency of HLA-B44/45 (the subtypes of the HLA-B12 antigen) is increased, but not significantly, so the HLA-B12 increase is probably secondary to that of HLA-DQw3.

Defence by the immune system against tumours induced by viruses is known to be important in experimental animals²¹⁻²³, and now our finding indicates that certain MHC genes could influence the development of squamous cell cervical carcinoma in humans. □

Received 17 May; accepted 1 July 1991.

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ACKNOWLEDGEMENTS. This work was supported by the Sanders Stiftung. We thank H. Graeff for blood samples of clinically diagnosed patients and H. Höfer for allowing us to use the histological diagnoses; I. Babaryka and E. Kaiditsch for tumour samples and histological advice; L. Butler and A. Wimmer for help with tissue typing and *in situ* hybridization; F. Zimmer, F. Fischbach and W. Machold for blood samples; and J. P. Johnson, D. J. Schendel and E. Simpson for monoclonal antibodies and for discussion.

Transport of cationic amino acids by the mouse ecotropic retrovirus receptor

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SUSCEPTIBILITY of rodent cells to infection by ecotropic murine leukaemia viruses (MuLV) is determined by binding of the virus envelope to a membrane receptor that has multiple membrane-spanning domains¹. Cells infected by ecotropic MuLV synthesize envelope protein, gp70, which binds to this receptor, thereby preventing additional infections. The consequences of envelope-MuLV receptor binding for the infected host cell have not been directly determined, partly because the cellular function of the MuLV receptor protein is unknown. Here we report a coincidence in the positions of the first eight putative membrane-spanning domains found in the virus receptor¹ and in two related proteins², the arginine²⁻⁴ and histidine^{2,3,5} permeases of *Saccharomyces cerevisiae* (Fig. 1), but not in any other proteins identified by computer-based sequence comparison of the GenBank data base¹. *Xenopus* oocytes injected with receptor-encoding messenger RNA show increased uptake of L-arginine, L-lysine and L-ornithine. The transport properties and the expression pattern of the virus receptor behave in ways previously attributed to y⁺ (refs 6, 7), the principal transporter of cationic L-amino acids in mammalian cells.

To examine the hypothesis that the ecotropic MuLV receptor might also transport arginine or histidine, we measured the uptake of ³H-labelled L-arginine and ¹⁴C-labelled L-histidine by *Xenopus* oocytes injected with mRNA prepared by *in vitro* transcription of the MuLV receptor complementary DNA cloned into pSP64T (ref. 8). Two days after injection, these oocytes

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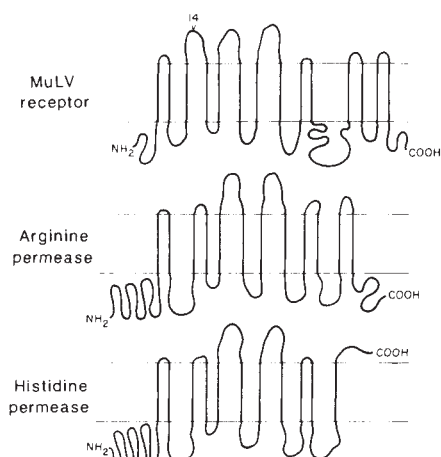
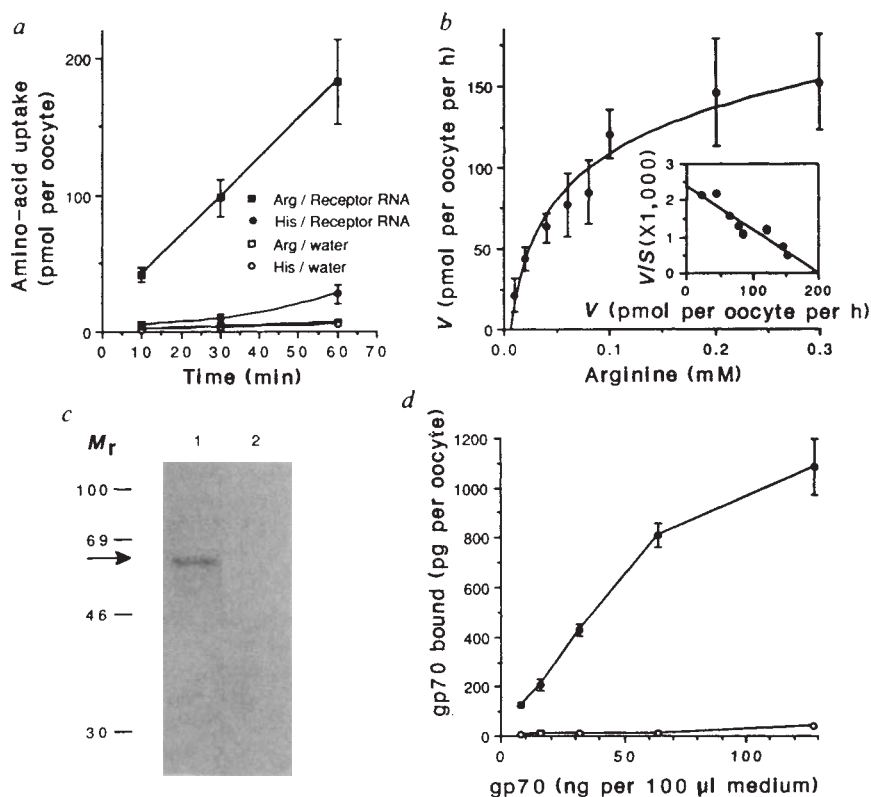


FIG. 1 A model demonstrating the relative positions of the putative membrane-spanning domains identified in the MuLV receptor and the yeast arginine and histidine permeases. These domains were previously assigned^{1,2}, and are depicted by vertical lines. The number of amino-acid residues between these domains is drawn to scale. The alignment between the three proteins has been determined by a computer-based algorithm²⁹ which optimizes sequence identity (and not the location of transmembrane domains). This alignment includes a 14-residue deletion in the first putative extracellular domain of the MuLV receptor (marked by arrow). There is a 19% amino-acid sequence identity between the MuLV receptor and the arginine permease and a 30% amino-acid sequence identity between the arginine and histidine permeases in this alignment.

FIG. 2 L-arginine transport into *Xenopus* oocytes injected with mRNA encoding the ecotropic MuLV receptor. *a*, Time course for uptake of L-arginine (squares) and L-histidine (circles) into oocytes injected with receptor mRNA (filled) or H₂O (open). Each point is the mean $\pm$ 1 s.d., $n=5$ oocytes. *b*, Kinetic analysis of L-arginine uptake into *Xenopus* oocytes injected with ecotropic receptor mRNA. Eadie-Hofstee plot is shown in the insert. $V_{\max}=190$ pmol per oocyte h⁻¹ (mean $\pm$ 1 s.d., $n=5$ oocytes). $K_m=0.07$ mM. *c*, The MuLV receptor protein (fused at the C' terminus to 25 residues of rat glucose transporter) identified by immunoprecipitation of ³⁵S-methionine-labelled oocytes using antibody to the C terminus of the rat glucose transporter and SDS PAGE. Migration of protein markers of known M_r ($\times 10^{-3}$) is marked. The oocytes were injected with mRNA prepared by *in vitro* transcription from the chimaeric MuLV receptor/glucose transporter cDNA described in Methods (lane 1) or H₂O (lane 2). *d*, Binding of ecotropic retrovirus envelope to oocytes injected with receptor mRNA (filled circles) or H₂O (open circles). Each point is gp70 bound (pg) $\pm$ 1 s.d., $n=5$ oocytes.

METHODS. *a, b*, The insert from the W1 cDNA (ref. 1) which encodes for the ecotropic retrovirus receptor protein was cloned into pSP64T (ref. 8). This plasmid, pSP64W1, was digested with *Bam*HI and RNA was prepared by *in vitro* transcription (Stratagene kit) using SP6 polymerase in the presence of additional GTP (0.25 mM) and mpppGTP (0.25 mM). Oocytes (Dumont, stage VI-VII) were excised from female *Xenopus laevis* anaesthetized by immersion in 3-aminobenzoic acid for 60 min. The oocytes were treated with collagenase II (Sigma) in calcium-free Barth's saline for 60 min., and incubated overnight in 50% Leibowitz's L-15 medium. The next day the oocytes were defolliculated with forceps, injected with RNA (5–50 ng in 50 nl H₂O) and incubated at 19 °C for 48 h. The oocytes were then placed in 0.3 ml uptake solution (NaCl 100 mM, KCl 2 mM, MgCl₂ 1 mM, CaCl₂ 1 mM, HEPES 10 mM, Tris 50 mM, pH 7.5) and L-arginine HCl (Sigma) 0.1 mM and 1.0 μ Ci of ³H-L-arginine (New England Nuclear) or L-histidine HCl (Sigma) 0.1 mM and 0.5 μ Ci ¹⁴C-L-histidine (New England Nuclear). After incubation, individual oocytes were washed 10 times in uptake solution, solubilized in SDS 1%, and counted in Aquasol. *c*, Ten *Xenopus* oocytes were injected with ³⁵S-methionine (1 μ Ci) (New England Nuclear) and RNA (50 ng) prepared by *in vitro* transcription from pSP64TW1-GT (*a*) or water (*b*). pSP64TW1-GT was constructed by substitution of bp 2,126–2,201 from pSPSM2 encoding the C'-terminal 25 amino acids of the rat glucose transporter protein¹⁰ for bp 2,027–2,425 from pSP64TW1 encoding



the C'-terminal 13 amino acids of the MuLV receptor protein¹ by polymerase chain reaction. RNA prepared by *in vitro* transcription from pSP64TW1-GT demonstrated unaltered L-arginine transport activity (data not shown). Six hours after injection, these oocytes were lysed in 50 μ l of 50 mM Tris, pH 7.5, 2 mM EDTA, 0.4% SDS, 1 mM PMSF and immunoprecipitation was performed using a rabbit antibody (1:100) raised against the C-terminal 35 amino-acid residues of the rat glucose transporter¹⁰. The washed Staph A pellets were recovered in 50 mM Tris, pH 7.5, 6 M urea, 2% SDS for SDS-PAGE and autoradiography. *d*, Ecotropic gp70 (30 μ g) was radiolabelled with Na ¹²⁵I using Iodobeads²¹ (Pierce). The labelled gp70 protein (10⁴ c.p.m. ng⁻¹) demonstrated specific binding to mouse NIH3T3 cells (data not shown). Oocytes were prepared as above and then incubated in 0.3 ml uptake solution +5% BSA and 25, 50, 100, 200 or 400 ng of ¹²⁵I-ecotropic gp70 envelope protein for 60 min at 19 °C. Oocytes were then washed three times in uptake solution +5% BSA before counting.

accumulated 27-fold more L-arginine in 1 h than controls injected with water (Fig. 2a). Uptake of L-histidine was increased less than twofold. Kinetic studies demonstrated that receptor-mediated uptake of L-arginine is saturable, indicating the existence of a carrier-mediated transport system (Fig. 2b). $V_{\max}$ calculated using the Eadie-Hofstee algorithm is variable between different oocyte preparations, but the $K_m = 0.07$ mM is reproducible and comparable to previously reported measurements ($K_m = 0.06$ – 0.145 mM) of L-arginine transport into murine hepatoma cells⁹. The expected protein of relative molecular mass 67,000 (M_r 67 K) could be identified by immunoprecipitation of ³⁵S-methionine-labelled oocyte lysates using an antibody against a C'-terminal epitope tag¹⁰ which can identify the translated product of the receptor-encoding mRNA (Fig. 2c). In addition, we observed 30-fold more binding of ¹²⁵I-labelled gp70 MuLV envelope to oocytes injected with virus receptor mRNA than to controls injected with water (Fig. 2d), an increase comparable to that for receptor-mediated L-arginine transport (Fig. 2a). Therefore, expression of a functional MuLV receptor protein in the oocyte membrane coincided with increased L-arginine uptake, a finding most consistent with a direct role for the receptor in L-arginine transport.

We then investigated the relationship between the MuLV receptor and a previously described pathway for cellular uptake of L-arginine which is independent of Na^+ in the incubation medium and is shared by the other cationic L-amino acids, L-lysine and L-ornithine^{6,7,9}. This pathway, termed y^+ (refs 6, 7), has been identified in fibroblast⁶ and hepatoma cell lines⁹,

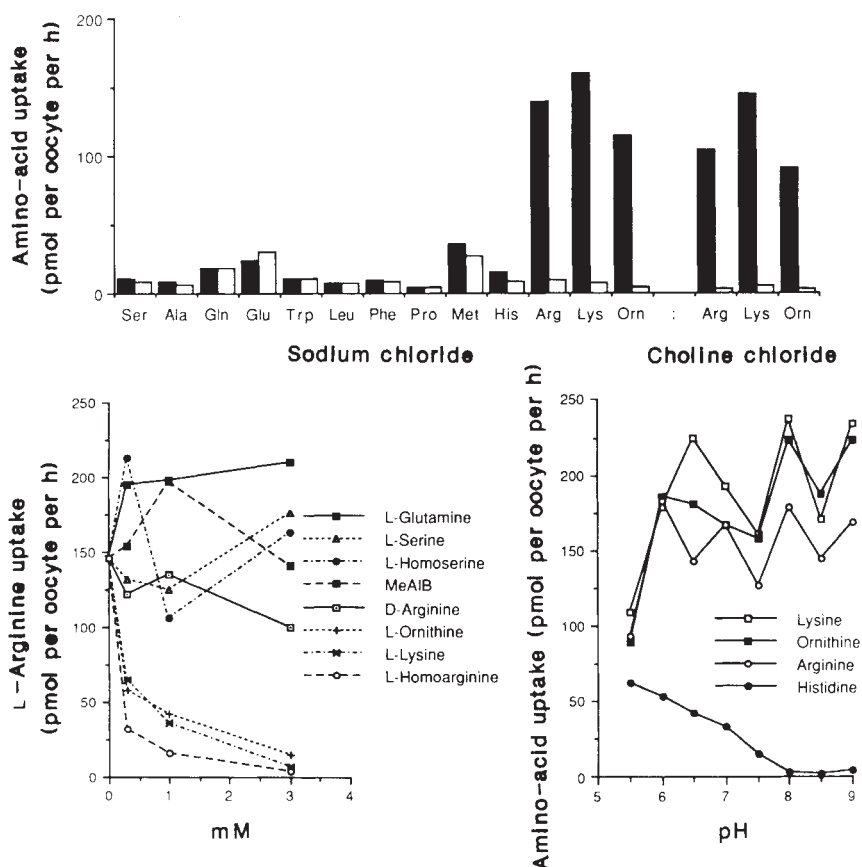
kidney^{11,12}, erythrocytes¹³ and embryos¹⁴, but not in liver^{7,9,15}. These properties distinguish y^+ from other transporters (A, ref. 16; ASC, ref. 17; N, ref. 18; L, ref. 19) that mediate uptake of neutral and acidic amino acids.

We did a survey of receptor-mediated uptake of amino acids into *Xenopus* oocytes and observed an increase in the uptake of L-lysine and L-ornithine which was comparable to L-arginine (Fig. 3a). There was no increase in uptake of L-serine, L-alanine, L-glutamine, L-glutamic acid, L-tryptophan, L-leucine, L-phenylalanine, L-proline, L-methionine or L-histidine, amino acids transported by mechanisms distinct from y^+ (refs 6, 7, 16–20). Furthermore, L-arginine uptake was inhibited by addition of increasing amounts of L-ornithine, L-lysine and L-homoarginine, but not L-serine, L-homoserine or L-glutamine, to the incubation medium, findings consistent with competition among the cationic amino acids for a shared transport mechanism (Fig. 3b). In addition, L-arginine transport was not inhibited by its stereoisomer, D-arginine, or by 2-(methyl)aminobutyric acid, a specific substrate for the system A transporter of neutral amino acids^{9,18}.

To further examine the apparent requirement of MuLV receptor-mediated amino-acid transport for a cationic side chain, we measured transport of L-histidine as a function of extracellular pH. Because the nitrogen on the imidazole side chain of L-histidine has a $pK_a = 6.1$, the majority of L-histidine molecules are cationic below this pH and may become suitable substrates for receptor transport. Indeed, we observed a 50-fold greater uptake of L-histidine by injected oocytes incubated at pH 5.5

FIG. 3 Amino-acid transport by the ecotropic MuLV receptor is specific for cationic L-amino acids and does not require extracellular Na^+ . **a**, Demonstration of the specificity for cationic amino acids and independence from Na^+ of ecotropic receptor-mediated amino-acid transport. Measurement of L-serine (Ser), L-alanine (Ala), L-glutamine (Gln), L-tryptophan (Trp), L-leucine (Leu), L-phenylalanine (Phe), L-proline (Pro), L-methionine (Met), L-histidine (His), L-arginine (Arg), L-lysine (Lys) and L-ornithine (Orn) uptake into oocytes injected with ecotropic receptor mRNA (black bar) or H_2O (white bar). Also shown is a measurement of L-arginine, L-lysine and L-ornithine uptake by *Xenopus* oocytes injected with receptor-encoding mRNA and incubated in uptake medium with substitution of choline chloride (100 mM) for NaCl (100 mM). Each bar is the mean of amino-acid uptake in five oocytes. **b**, Inhibition of MuLV receptor-mediated L-arginine transport by increasing concentrations of L-ornithine, L-homoarginine and L-lysine, but not L-glutamine, L-serine, L-homoserine, 2-(methyl)aminobutyric acid or D-arginine. Each data point is the mean uptake of L-arginine from three oocytes. **c**, MuLV receptor-mediated transport of L-histidine as a function of extracellular pH. Each data point is the mean of L-histidine uptake from three oocytes.

METHODS. Oocytes were prepared and injected as in Fig. 2a. **a**, Two days later, individual oocytes were washed and counted after a 1 h incubation in uptake media containing 1.0 μCi per 0.1 mM ³H-labelled L-serine, L-alanine, L-glutamine, L-glutamic acid, L-tryptophan, L-leucine, L-phenylalanine, L-proline, L-methionine, L-arginine, L-lysine or 0.5 μCi per 0.1 mM ¹⁴C-labelled L-histidine or L-ornithine. In separate experiments, uptake of 1.0 μCi per 0.1 mM ³H-labelled L-arginine, L-lysine, or 0.5 μCi per 0.1 mM ¹⁴C-labelled L-ornithine into injected oocytes was measured in uptake medium modified by substitution of 100 mM choline chloride for 100 mM NaCl. **b**, Uptake of 1.0 μCi per 0.1 mM ³H-labelled L-arginine measured in oocytes injected with 50 nl MuLV receptor mRNA (1 ng nl⁻¹) and incubated in medium containing 0, 0.3, 1.0 or 3.0 mM unlabelled L-homoarginine, L-lysine, L-ornithine, L-homoserine, L-serine, L-glutamine, 2-



(methyl)aminobutyric acid or D-arginine. **c**, Uptake of 1.0 μCi per 0.1 mM ³H-labelled L-arginine, ³H-labelled L-lysine or 0.5 μCi per 0.1 mM ¹⁴C-labelled L-histidine, ¹⁴C-labelled L-ornithine into oocytes injected with 50 nl receptor mRNA (1 ng nl⁻¹) was measured in uptake medium buffered with 10 mM sodium acetate, pH 5.5, 10 mM PIPES, pH 6–7, or 10 mM Tris, pH 8–9. pH was adjusted with acetic acid or hydrochloric acid.

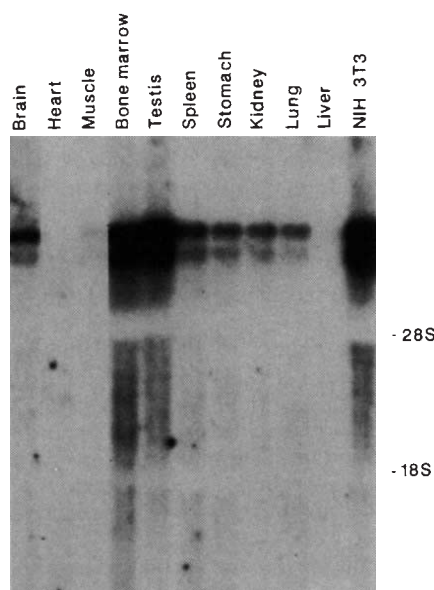


FIG. 4 Expression of the ecotropic retrovirus receptor gene in mouse tissues. A northern blot prepared using RNA from tissues obtained from a 3-month-old FVB mouse and mouse NIH3T3 cells was hybridized to a ^{32}P -labelled receptor probe and exposed to film for 9 days. Mouse cells contain two receptor transcripts (7.9 and 7.0 kb) which result from use of alternative polyadenylation sites¹. The migration position of the 28S and 18S ribosomal RNAs are indicated. Additional hybridizations using radiolabelled probes derived from the *Tea*³⁰ and rat actin genes demonstrated comparable integrity and quantity of the RNA prepared from liver and other tissues. METHODS. RNA was purified from indicated tissues and 10 μg of each RNA was separated on an agarose-formaldehyde gel, blotted onto nitrocellulose and hybridized to the ^{32}P -labelled pSP64W1 insert using methods previously reported⁴. The blot was washed ($0.1 \times \text{SSC}$, 65°C) and autoradiographed.

than at pH 8 (Fig. 3c). This pH-dependent increase in L-histidine uptake is consistent with a preference of the MuLV receptor for cationic substrates, but is less than predicted by the titration of the L-histidine side chain. This difference may be a consequence of pH-dependent effects upon the MuLV receptor or constraints on L-histidine transport conferred by the imidazole ring. In addition to the importance of a cationic side chain, the failure of D-arginine to inhibit competitively the transport of its stereoisomer (Fig. 3b) suggests that both the α -amino group and the carboxyl group of the amino acid must also contribute to the specificity of receptor-mediated transport. Taken together, these results demonstrate that the ecotropic MuLV receptor is a transporter which is specific for cationic L-amino acids.

In addition, receptor-mediated uptake of L-arginine, L-lysine and L-ornithine into oocytes does not require Na^+ in the incuba-

tion medium (Fig. 3a) and the MuLV receptor transcripts could not be detected in liver RNA (Fig. 4). Others have reported the failure of ecotropic retroviruses to infect mouse hepatocytes^{21,22}, an observation which may be explained by the absence of virus receptors on these cells. Examination of RNA prepared from other mouse tissues by northern blot identified the greatest expression of the 7.9-kilobase (kb) and 7.0-kb MuLV receptor transcripts in testes, a tissue which synthesizes large amounts of arginine- and lysine-rich proteins such as protamine and histones. The receptor transcripts were also present in RNA prepared from bone marrow, brain, skeletal muscle, heart, stomach, spleen, kidney and lung (Fig. 4).

Both the competition experiments (Fig. 3b) and the pH dependence of L-histidine transport (Fig. 3c) support our initial observation that cationic amino acids such as L-arginine, L-lysine and L-ornithine, are specific substrates for transport by the MuLV receptor. In addition, the independence of MuLV receptor-mediated cationic amino-acid transport from extracellular Na^+ and the absence of expression in liver are properties which are not found together in transporters of other amino acids^{7,16-19}. Therefore, the behaviour of the MuLV receptor as an amino-acid transporter cannot be distinguished from y^+ , the principal pathway for uptake of cationic L-amino acids by mammalian cells. Furthermore, the structural and functional relationship between the MuLV receptor and yeast arginine and histidine permeases suggests that closely related proteins which transport cationic amino acids may be found in other eukaryotes.

In addition to y^+ , a Na^+ -dependent transporter of lysine, arginine and cystine has been identified in the apical brush border of the renal proximal tubule^{23,24} and the jejunum²⁵. Also, there is transport of L-ornithine into the mitochondria of hepatocytes²⁶, cells which do not express the virus receptor. These examples of tissue-specific uptake of cationic L-amino acids may be explained by the existence of additional transporters which are related to the MuLV receptor, a finding which would be analogous to the previously identified regulation of glucose uptake by a family of related transporters^{10,27,28}.

Inhibition of cationic amino-acid transport by neutral amino acids such as glutamine and serine has been reported in cell-culture experiments^{6,7}. It is unlikely that these findings can be explained by an overlap in the substrate specificity of the cationic amino-acid transporter, as has been proposed, as this finding was not observed in MuLV receptor-mediated transport in *Xenopus* oocytes (Fig. 3b). It remains possible that there is an interaction between the MuLV receptor and other amino-acid transporters in mouse cell membranes.

These findings raise the possibility that changes in the nutritional or hormonal state of the host which influence amino-acid metabolism could alter receptor expression and thereby change susceptibility to ecotropic retrovirus infection. In addition, they permit direct examination of the consequences of envelope gp70-receptor interaction for infected mouse cells. □

Received 20 May; accepted 1 July 1991.

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ACKNOWLEDGEMENTS. We thank R. Smith for performing the computer analysis, M. Birnbaum for supplying glucose transporter clones and antibody, C. MacLeod for supplying the p20.5 clone, M. Berry and M. Birnbaum for advice on oocyte injection, C. Kelly for technical assistance, R. Weinberg and E. Kieff for criticism of the manuscript and P. Gould for help in its preparation. E.I.C. was supported by a grant from the Deutsche Forschungsgemeinschaft. This work was supported by the Howard Hughes Medical Institute and the NIH (to J.M.C.).

Cell-surface receptor for ecotropic murine retroviruses is a basic amino-acid transporter

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THE complementary DNA sequence encoding the cell-surface receptor for ecotropic host-range murine retroviruses (ecoR) shows that it contains 622 amino acids and 14 hydrophobic potentially membrane-spanning sequences¹. Because this receptor occurs on many or all murine cells² and is probably essential for viability of cultured fibroblasts³, its normal function might be to transport an essential metabolite. We expressed ecoR in *Xenopus laevis* oocytes by injecting RNA transcribed from the cloned cDNA. These oocytes specifically bound the gp70 envelope glycoprotein from an ecotropic murine leukaemia virus. An inward current was recorded electrophysiologically when a mixture of amino-acids was applied: this resulted from a stereoselective, saturable uptake of lysine, arginine and ornithine; it was independent of sodium and not substantially altered by gp70. Cysteine and homoserine were also taken up, but sodium was necessary for their transport. These properties of ecoR correspond to those of the y⁺ amino-acid transporter⁴⁻⁶. Our results demonstrate the subversion of a ubiquitous cell membrane protein, in this case a basic amino acid transporter, for use as a retroviral receptor.

EcoR RNA was transcribed from the cDNA and injected into *Xenopus* oocytes⁷. Expression of ecoR was detected 2-6 days after injection by incubating the oocytes with gp70 that had been purified from ecotropic murine leukaemia virus (MuLV-E), followed by incubation with goat antiserum raised against gp70 and then with [¹²⁵I]-labelled protein A (refs. 3, 8). Oocytes injected with ecoR messenger RNA bound substantially more gp70 than uninjected oocytes (Fig. 1a). In addition, the oocytes that had adsorbed gp70 and antibody were specifically lysed by incubation with rabbit complement (Fig. 1b)^{3,8}.

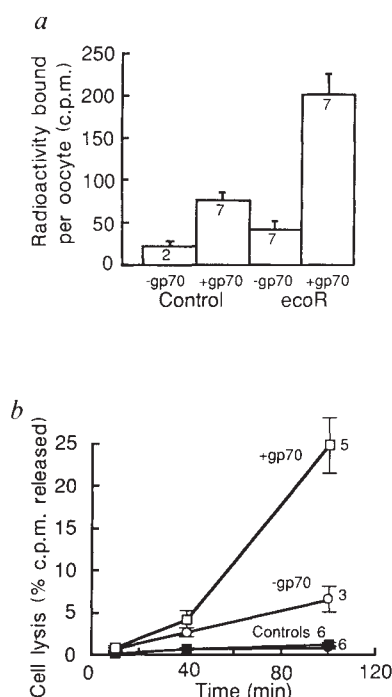


TABLE 1 Values of K_m and I_{max} from current measurements

Amino acid (mM)	K_m (mM)	I_{max}
Arginine (0.01–10)	0.077 ± 0.002	0.98 ± 0.02
Lysine (0.01–3)	0.073 ± 0.008	0.95 ± 0.02
Ornithine (0.01–3)	0.105 ± 0.002	1.17 ± 0.03
D-arginine (0.1–10)	1.82 ± 0.15	1.75 ± 0.04
D-lysine (1–30)	21.1 ± 4.3	0.99 ± 0.15
Cysteine (1–30)*	24.7 ± 2.1	1.56 ± 0.07
Histidine (0.01–10)†	1.83 ± 0.07	2.02 ± 0.06
Homoserine (10)*‡		0.67 ± 0.04

K_m and I_{max} determined from current measurements in the way shown in Fig. 2c. Amino acids are L-isomers except where stated.

* No current in sodium-free solution.

† Current induced by histidine (10 mM) was reduced by $59 \pm 4\%$ ($n=3$) in sodium-free solution.

‡ Only one concentration applied; current is fraction of that caused by 10 mM arginine in same oocytes. Glutamine, glutamate, asparagine, aspartate, glycine, proline and serine were each applied at 1 mM; they neither evoked a current nor blocked the action of lysine.

Inward currents of 50–100 nA were detected in injected oocytes when an amino-acid mixture (modified Eagle's medium, GIBCO) was applied. This was found to result from a saturable uptake of basic amino acids. Figure 2 shows measurements made with a two-electrode voltage clamp at -60 mV. Lysine induced a concentration-dependent inward current that reached a maximum at ~ 1 mM. Arginine, ornithine and histidine gave similar currents, but several neutral or negatively charged amino acids had no effect (Table 1). The maximal current and the Michaelis constant, K_m , were estimated by fitting from hyperbolic functions (Fig. 2c) and the results are summarized in Table 1. The current became larger with hyperpolarization and smaller with depolarization, but was still inward at $+20$ mV. Uninjected oocytes showed little (< 5 nA) or no response to lysine, arginine, ornithine, histidine, homoserine or cysteine (all at 10 mM).

System y⁺ is a sodium-independent transporter for basic amino acids that has been extensively characterized^{4-6,9-11}. In

FIG. 1 Binding of gp70 and complement-induced cell lysis in *Xenopus* oocytes expressing ecoR. a, Binding of [¹²⁵I]-labelled protein A to oocytes previously injected with ecoR RNA or uninjected controls. Oocytes were incubated either with (+gp70) or without (–gp70) gp70 before addition of anti-gp70 antiserum followed by [¹²⁵I]-labelled protein A. Bars represent s.e.m. b, Complement-induced lysis of oocytes previously injected with ecoR RNA (open symbols) compared with uninjected controls (closed symbols). Oocytes in each group were preloaded for 2 h with Tran³⁵S-label (ICN Biomedicals) followed by a 2-h incubation with (squares) or without (circles) gp70. All oocytes were then incubated with antiserum to gp70 in the presence of rabbit complement. The total radioactivity (released by complement plus that remaining) was not different for the four groups.

METHODS. The ecoR cDNA was kindly provided by J. Cunningham. A 2.3-kb pair BamHI–EcoRI restriction enzyme fragment was removed from pJET and cloned into pGEM3 vector to form pGEM3–ecoR; this was linearized with EcoRI for *in vitro* transcription. Transcription was carried out as described⁷. Oocytes were injected with 5–10 ng ecoR RNA and maintained for 2–6 days⁷. For gp70 binding, oocytes were incubated for 2 h with purified gp70 ($2 \mu\text{g ml}^{-1}$), for 1 h with 1:200 goat anti-gp70 antiserum, and for 1 h with $5 \mu\text{Ci}$ of [¹²⁵I]-labelled protein A in 1 ml ND-96 solution⁷. Bound radioactivity was counted for individual oocytes after three 10-min washes with 2 ml salt solution ND-96 (ref. 7). The cytotoxicity assay was carried out by labelling three to six oocytes for 2 h with Tran³⁵S ($10 \mu\text{Ci ml}^{-1}$) followed by 2 h incubation in nonradioactive ND-96, three washes in ND-96, 2 h incubation with or without gp70 ($2 \mu\text{g ml}^{-1}$) and three further washes with ND-96. Goat anti-gp70 antiserum (1:200) and rabbit complement (1:10; GIBCO) were then added. Supernatant (20 μl) was taken for scintillation counting at 10, 40 and 100 min; total radioactivity was then measured from oocytes lysed by forcing them through a small pipette tip in 0.1% sodium dodecyl sulphate.

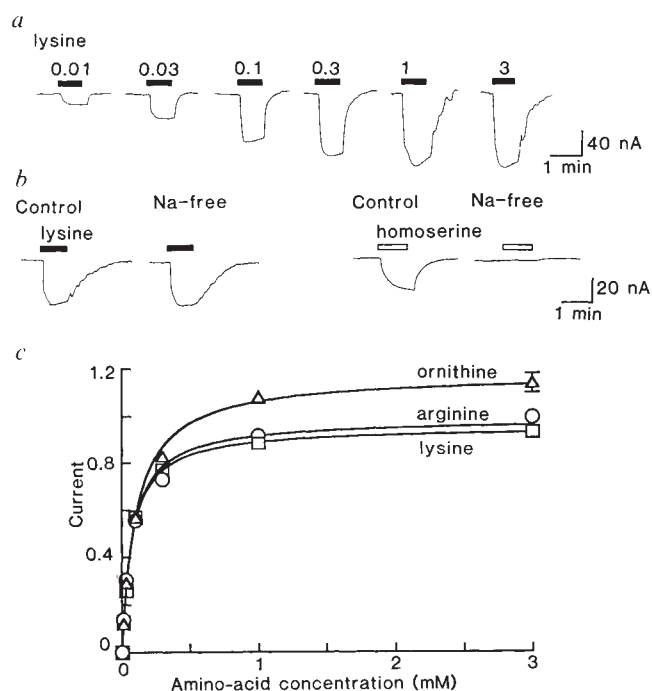


FIG. 2 *a*, Inward currents evoked by lysine in *Xenopus* oocyte previously injected with *ecoR* RNA. Lysine concentrations are indicated above each record (mM); bars indicated period of application. Holding potential was -60 mV. *b*, Current induced by lysine (1 mM) is sodium-independent. A similar result was obtained for arginine. Homoserine (10 mM; application indicated by open bars) induces a sodium-dependent inward current. Currents shown in *b* are from same oocyte; holding potential -60 mV. Sodium-free solution was substituted with equimolar Tris. *c*, Current induced by different concentrations of arginine, lysine and ornithine. Peak currents (I) evoked by a given concentration were measured. The currents were normalized to the current evoked by 10 mM arginine in the same oocyte, which ranged from 70–120 nA. Points are mean $\pm$ s.e.m. ($n=3-4$ oocytes). Lines shown are fitted by least-squares to a function of the form $I = (I_{\max}[\text{amino acid}]) / (K_m + [\text{amino acid}])$.

METHODS. Two-electrode voltage-clamp recordings were made from *Xenopus* oocytes⁷. All experiments were at room temperature, and currents illustrated are in cells clamped at -60 mV. Amino acids were applied by changing the perfusing solution to one that contained the amino acid.

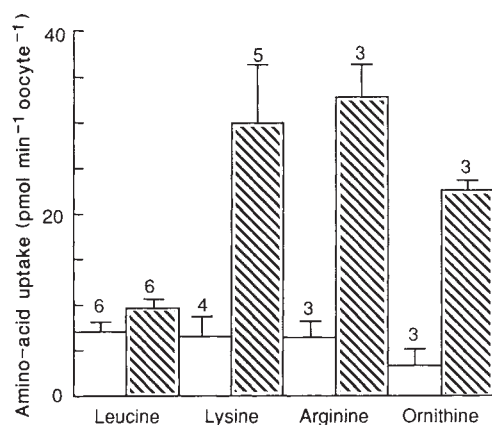


FIG. 3 Uptake of tritiated amino acids by oocytes expressing *ecoR*. Uptake of leucine, lysine, arginine and ornithine is shown for groups of oocytes that had been injected with *ecoR* RNA (cross-hatched) or uninjected controls (open). Vertical bars give s.e.m. for number of oocytes indicated above each column.

METHODS. Individual oocytes were incubated for 3 min in ND-96 (1 ml) that contained 200 μ M of 3 H-labelled amino acid. Individual oocytes were then lysed and radioactivity was counted by liquid scintillation. L-[4,5- 3 H]leucine and L-[4,5- 3 H]lysine were from Amersham; L-[2,3- 3 H]arginine and L-[2,3- 3 H]ornithine were from New England Nuclear; final specific activity was 50 μ Ci μ mol⁻¹.

oocytes expressing *ecoR*, the current induced by lysine is also sodium-independent (Fig. 2*b*). One characteristic of system y^+ is that the transport of basic amino acids is inhibited by some neutral amino acids, such as homoserine, but only in the presence of sodium^{6,9-11}. Homoserine itself induced an inward current in injected oocytes, but this was completely dependent on the presence of extracellular sodium (Fig. 2*b*). Thus, *ecoR* transports basic amino acids in a sodium-independent manner and some neutral amino acids by a sodium-dependent process (Table 1).

Consistent with our electrical measurements and the properties of the system y^+ transporter, individual oocytes expressing *ecoR* rapidly accumulated [3 H]lysine, [3 H]arginine and [3 H]ornithine when they were added at 200 μ M (Fig. 3). The uptake is linear during the first 3 min, being $\sim 25-30$ pmol min⁻¹. The uptake of [3 H]leucine is no greater in oocytes injected with *ecoR* than in control oocytes, and is comparable to the background uptake of the other amino acids in uninjected oocytes (Fig. 3). There is good agreement between the uptake measured radiochemically and that measured electrophysiologically (30 pmol min⁻¹ corresponds to 48 nA for univalent ions) (Figs 2 and 3).

Addition of gp70 (2 μ g ml⁻¹; 4 h) or intact Moloney MuLV (20 μ g ml⁻¹; 4 h) at 25 $^{\circ}$ C did not alter amino-acid transport by *ecoR*. Thus, L-arginine (10 mM) evoked currents of 54.4 ± 7.8 nA in control cells, 58.0 ± 8.5 nA in gp70-treated cells and 57.2 ± 5.9 nA in virus-treated cells (five oocytes in each case from the same batch). This suggests that virus binding to *ecoR* might not perturb transport, but we emphasize that this is only a preliminary conclusion because there are several time- and temperature-dependent stages in virus adsorption onto cells^{2,3,8,12}. We also have to determine whether substrates for uptake, or possibly inhibitors of uptake, interfere with gp70 adsorption and/or viral infection.

Our results identify this retrovirus receptor as the sodium-independent basic amino-acid uptake system generally known as y^+ (refs 4–6, 9–11): the K_m s determined here agree with those reported for system y^+ in several other cells, there is selectivity of L-amino acids over D-amino acids, and homoserine transport is dependent on Na⁺ (Table 1). Previous findings are consistent with this conclusion. Both system y^+ (ref. 6) and *ecoR* (refs 2, 23) have a ubiquitous tissue distribution. Furthermore, the predicted transmembrane topology of *ecoR* is homologous to other known membrane transport proteins that have 12–14 hydrophobic domains¹; these include the products of the yeast genes CAN1 (ref. 13), CTR (ref. 14) and HIP1 (ref. 15), which are arginine, choline and histidine transporters, respectively. Knowing the molecular structure of this family of transporters should help our understanding of those inherited human diseases that result from a defective transport of basic amino acids¹⁶.

These results also raise issues pertaining to *ecoR* function in retrovirus infections. Interference enables a cell infected by a retrovirus to resist superinfection by any retrovirus of the same host-range class^{2,17–21}. It is important to find out whether the receptor blockade or downregulation associated with interference^{2,17–21} perturbs *ecoR* amino-acid transport. Ecotropic MuLV envelope glycoproteins have been implicated not only in leukaemogenesis^{2,22–24}, but in immunosuppression^{25–27}, haemolytic anaemia²⁸ and neuronal degeneration²⁹, and it is possible that these involve *ecoR* transport abnormalities.

A cDNA has been isolated from T-lymphoma cells that encodes a protein (Tea) which is highly homologous to *ecoR*³⁰, and might also be a transporter. Another cDNA that encodes a cell-surface receptor for Gibbon ape leukaemia virus was recently cloned³¹, and although the expected protein does not share sequence homology with *ecoR*, it is also very hydrophobic and has a similar number of potential membrane-spanning domains. These features suggest that this other receptor for a C-type retrovirus might also be a transport protein. □

Received 4 June; accepted 2 July 1991.

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ACKNOWLEDGEMENTS. Supported by the NIH. We thank J. Cunningham (Harvard Medical School) for the *ecoR* cDNA and Yan-Na Wu for injecting oocytes.

Basal body movements as a mechanism for mitochondrial genome segregation in the trypanosome cell cycle

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THE mitochondrial genome of *Trypanosoma brucei* is organized in the form of a complex catenated network of circular DNA molecules. This mass of DNA, known as the kinetoplast, is present at a unique site in the single mitochondrion, and is replicated in a discrete, periodic S phase of the cell cycle. The single-copy nature of the kinetoplast suggests that there is a mechanism ensuring segregation fidelity of replicated copies to each daughter cell. Historically, speculation regarding the nature of this mechanism has often attributed significance to the close association between the kinetoplast and the flagellum basal body. We provide here direct evidence that this mitochondrial DNA complex is indeed linked to the basal body, and segregation of the kinetoplast DNA is dependent on a microtubule-mediated separation of the new and old flagellar basal bodies during the cell cycle. This unique system may represent the remnants of an evolutionarily archaic mechanism for genome segregation.

Light-microscope descriptions of the trypanosome cell comment on the coincidence of location of the flagellar basal bodies and the kinetoplast. Phase-contrast microscopy illustrates this architecture showing an elongated trypanosome cell with a single flagellum (Fig. 1a); the single nucleus and kinetoplast are revealed by DAPI staining (Fig. 1b). Electron microscopy reveals that the mitochondrion runs the length of the elongated

cell and that the kinetoplast is always next and orthogonal to the basal body (Fig. 1c)¹.

In a definitive description of haematoxylin-stained trypanosome cells, Robertson observed these relationships², although the precise identity of the structures was not known. She noted the structural coincidence of the two organelles and discussed the possibility that one may exert a segregational effect on the other during cell morphogenesis. Many authors have subsequently reiterated this hypothesis^{3–8}. Although there has been considerable improvement in our understanding of the function and organization of these organelles there is still little insight into how the trypanosome cell accomplishes the segregation of these single-copy organelles during cell division. We have used specific probes to locate each organelle, and when combined with drug intervention experiments these reveal the nature of the segregation process.

We labelled trypanosome cells with two monoclonal antibodies which particularly allow detection of both the flagellum and the basal bodies. We chose the ROD1 (ref. 9) antibody as it detects the paraflagellar rod (a structure that runs alongside the flagellar axoneme) at the point of its emergence from the cell to the flagellar tip. This leaves an unlabelled region between the proximal end of the paraflagellar rod and the basal bodies, allowing simultaneous visualization of the basal bodies by the monoclonal antibody BBA4 (ref. 9). These probes reveal the high-order precision of organelle position and replication during the cell cycle. It is possible to determine the position of a cell in the cell cycle by scoring criteria such as the presence of a new flagellum, the relative length of old and new flagella and the distance apart of basal bodies (Fig. 2a, c, e)¹⁰. DAPI staining of these cells also reveals the position both of the nuclei and of the kinetoplasts. (Fig. 2b, d, f).

To reveal dependency relationships between these movements we applied these probes to cells after treatments with drugs designed to compromise organelle segregation. First, we asked whether inhibition of the segregation of kinetoplast DNA affects basal body segregation. As the kinetoplast is a complex mass of catenated DNA molecules we reasoned that inhibitors of topoisomerase II should jeopardize segregation by inhibiting the decatenation–catenation mechanisms^{11–13}. Independent application of two drugs, teniposide and ethidium bromide, to trypanosome cultures for periods just under the cell doubling time resulted in the appearance of cells that possessed stretched

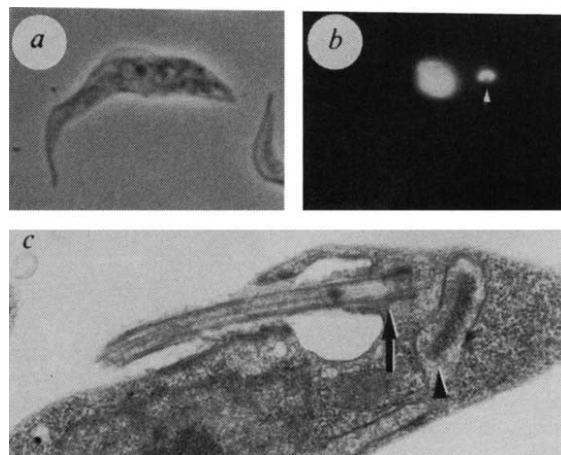


FIG. 1 Visualization of the intimate association between flagellar basal bodies and the mitochondrial genome in *T. brucei*. a, Phase-contrast micrograph of a culture form trypanosome. b, Identical cell stained with the DNA-intercalating dye DAPI (4,6-diamidino-2-phenylindole; Sigma) reveals the large nucleus and the smaller kinetoplast. c, Electron micrograph of a trypanosome sectioned longitudinally, displaying orthogonal alignment of kinetoplast with the flagellum basal body. c, Arrow indicates the basal body; b and c, arrowheads indicate kinetoplasts.

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masses of kinetoplast DNA (Fig. 3*b, d*). Immunofluorescence using the two monoclonal antibody probes shows that such cells have relatively long new flagella and that the basal bodies have separated essentially as normal. Interestingly, the basal bodies are located at the poles of these stretched kinetoplast structures, (Fig. 3*a, c*). Thus, although this treatment compromises the segregation of the kinetoplast DNA, it does not seem to affect basal body segregation.

Second, does the separation of basal bodies influence kinetoplast segregation? We used a concentration of the antimicrotubule drug ansamitocin ($2\text{ }\mu\text{M}$), which halts basal body separation by inhibiting microtubule cytoskeletal morphogenesis¹⁴. This concentration of ansamitocin does not seriously affect growth of the new flagellar axoneme. Examination of cultures after such drug treatment revealed cells with the unusual morphology seen in Fig. 3*e* and *f*. Such cells have produced long new flagella and yet the basal bodies of the old and new flagella have not moved apart. The cell in Fig. 3*e* is such an example in that it has a relative flagella length ratio (new to old) of 0.47. In a control cell this should signify that the basal bodies would be separated by about $2.7\text{ }\mu\text{m}$. In fact, the basal bodies are still juxtaposed (Fig. 3*e*). Although the kinetoplast DNA complexes have replicated, as represented by an approximate duplication of DAPI staining, they have not segregated (Fig. 3*f*).

We interpret the above experiments as providing direct evidence that the basal bodies do indeed mediate the efficient

segregation of the mitochondrial genome complexes in *T. brucei*. Such a mechanism therefore suggests the presence of some form of link between the two organelles. At its most extreme such a link might be envisaged as 'molecular hard wiring' between the mitochondrial matrix and a cytoplasmic organelle. We have tested the possibility that such a connection exists by isolating flagella and showing that kinetoplast DNA remains attached to their proximal (basal body) ends. First, we have used a protocol which involves hypotonic lysis and depolymerization of the subpellicular microtubule array by the addition of a high concentration of Ca^{2+} . This results in a population of flagella-kinetoplast complexes, with the kinetoplasts still tightly associated with the basal bodies (Fig. 4*a*). Second, we extracted cells with a buffer containing Triton X-100, a nonionic detergent which solubilizes membrane components, leaving a cytoskeleton. Again, solubilizing the subpellicular microtubule corset allows isolation of a flagella preparation exhibiting attached kinetoplast DNA complexes (Fig. 4*b*), thus suggesting that membranous structures are not an absolute requirement for maintenance of this unique association.

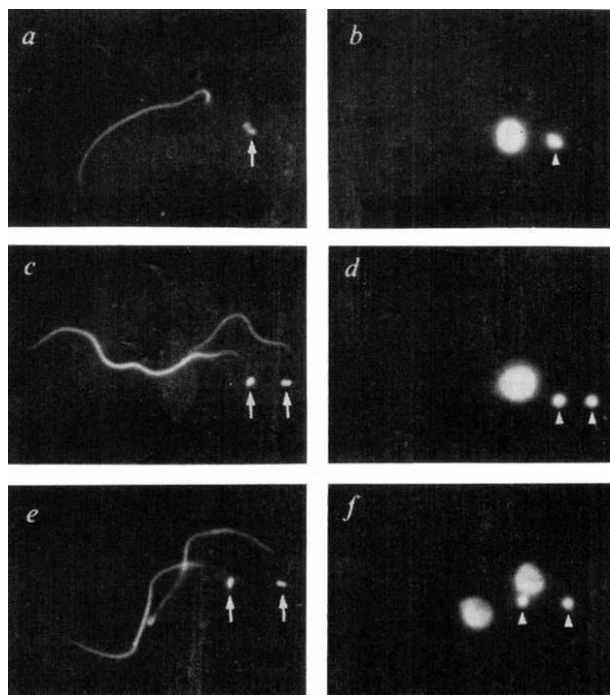


FIG. 2 Flagella and kinetoplast organization in the cell cycle of *T. brucei*. *a, c* and *e*, Photomicrographs of trypanosomes labelled with ROD1 and BBA4 monoclonal antibody probes, viewed using fluorescence microscopy. Note the development of the new flagellum coincides with the separation of the replicated kinetoplasts, seen when viewed after DAPI staining *b, d* and *f*. Arrows indicate basal bodies; arrowheads indicate kinetoplasts.

METHODS. Asynchronous early to mid-log phase culture cells ($3\text{--}4 \times 10^6\text{ ml}^{-1}$ *T. brucei* strain 427), were grown at 28°C in SDM79 supplemented with 10% fetal calf serum¹⁵, and collected by centrifugation at $1,000g$ for 10 min. Immunofluorescence is essentially as in ref. 16. Briefly, cells are washed in PBS (pH 7.4), settled on slides, fixed in methanol at -20°C for 60 min, rehydrated in PBS, and incubated with a 1:1 mixture of mouse monoclonals ROD1 and BBA4 for 60 min. After incubation cells are washed, incubated in rabbit anti-mouse FITC conjugate diluted 1:40 in PBS, stained with DAPI, mounted in Mowiol 4-88 and viewed by phase contrast and fluorescence microscopy.

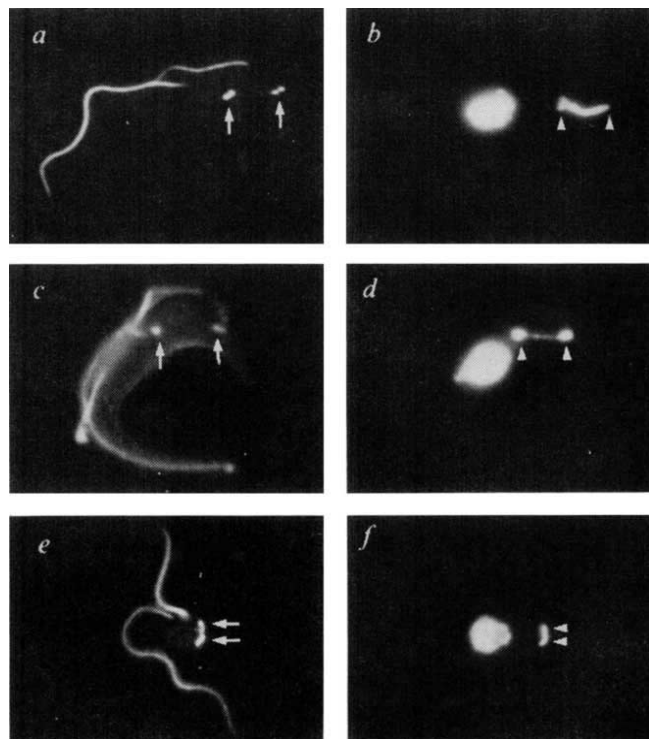


FIG. 3 Drug treatment of cultured trypanosomes reveals processes involved in kinetoplast segregation. *a*, Immunofluorescence photomicrograph of a cell treated for 8 h with ethidium bromide, final concentration of $500\text{ }\mu\text{M}$, and labelled with ROD1 and BBA4. *b*, Same cell as in *a*, viewed after DAPI staining. The kinetoplast DNA is stretched between the separated basal bodies. *c, d*, A cell treated with teniposide for 8 h, final concentration of $200\text{ }\mu\text{M}$. Antibody labelling and DAPI staining as *a*. Note again that 'normal' kinetoplast segregation is impeded. *e, f*, Immunofluorescence photomicrographs of a cell treated with ansamitocin for 7 h, final concentration $2\text{ }\mu\text{M}$. Antibody labelling and DAPI staining as *a*. Basal body and kinetoplast segregation has been halted. Arrows indicate basal bodies, arrowheads indicate kinetoplasts.

METHODS. Culture form *T. brucei*, strain 427, are grown to early mid-log phase $3\text{--}4 \times 10^6\text{ ml}^{-1}$ at 28°C as previously described. Topoisomerase II poisons: ethidium bromide solubilized in SDM79 was added to the culture to a final concentration of $500\text{ }\mu\text{M}$ for 8 h at 28°C . The cells are then washed twice in PBS and treated for immunofluorescence. Teniposide solubilized in DMSO (dimethyl sulphoxide) was added to a culture, final concentration of $200\text{ }\mu\text{M}$ for 8 h at 28°C . The cells are then washed twice in PBS and treated for immunofluorescence. Microtubule poisons: ansamitocin solubilized in DMSO was added to a culture to a final concentration of $2\text{ }\mu\text{M}$ for 7 h. The cells are washed twice in PBS, and treated for immunofluorescence.

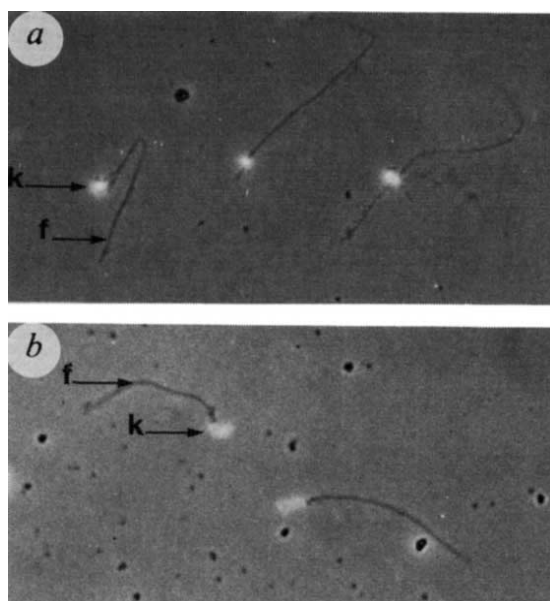


FIG. 4 Combined phase-contrast and DAPI fluorescence micrographs of isolated *T. brucei* flagella show kinetoplasts (bright fluorescent dots) still attached to basal bodies. *a*, Flagella isolated by hypotonic lysis and cytoskeletal depolymerization. *b*, Flagella isolated by detergent lysis and cytoskeletal depolymerization. The isolated flagella (f) and DAPI stained kinetoplasts (k) are viewed by combined phase-contrast/fluorescence microscopy.

METHODS. Hypotonic lysis: log-phase cell cultures were collected by centrifugation, washed three times in 0.25 M sucrose, 20 mM Tris-HCl, pH 7.9. Microtubule cytoskeletal depolymerization and cell lysis was achieved by resuspending and incubating cells in 3 mM Ca^{2+} in double distilled water for 3 h at 4 °C. Detergent lysis: EDTA was added to a mid-log phase culture, $4\text{--}5 \times 10^6$ cells ml^{-1} , final concentration of 5 mM. Cells were then collected by centrifugation, washed in PBS, resuspended on ice for 10 min in extraction buffer, (0.5% Triton X-100 in PMN (10 mM NaH_2PO_4 , 150 mM NaCl, 1 mM MgCl_2 pH 7.2)). Cytoskeletons were collected by centrifugation, washed in extraction buffer, and resuspended on ice for 45 min in 1 mM Ca^{2+} in PMN. Isolated flagella are DAPI-stained and viewed by fluorescence microscopy.

Our experiments reveal an established structural and functional link between the mitochondrial genome and basal bodies in the *T. brucei* cell. This link provides a means of ensuring high fidelity segregation of these single-copy cytoplasmic organelles to daughter cells. By contrast, other eukaryotic cells possess multiple and dispersed mitochondrial genomes which may not require such stringent segregation fidelity. The high-order system present in *T. brucei* may represent a remnant of an evolutionarily archaic mechanism for DNA segregation. □

Cloning of an NF- κ B subunit which stimulates HIV transcription in synergy with p65

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THE transcription factor NF- κ B is a protein complex which comprises a DNA-binding subunit and an associated transactivation protein (of relative molecular masses 50,000 (50K) and 65K, respectively)^{1,2}. Both the 50K and 65K subunits have similarity with the *rel* oncogene and the *Drosophila* maternal effect gene *dorsal*³⁻⁶. The 50K DNA-binding subunit was previously thought to be a unique protein, derived from the 105K gene product (p105). We now report the isolation of a complementary DNA that encodes an alternative DNA-binding subunit of NF- κ B. It is more similar to p105 NF- κ B than other family members and defines a new subset of *rel*-related genes. It is synthesized as a ~100K protein (p100) that is expressed in different cell types, contains cell cycle motifs and, like p105, must be processed to generate a 50K form. A 49K product (p49) can be generated independently from an alternatively spliced transcript; it has specific κ B DNA-binding activity and can form heterodimers with other *rel* proteins. In contrast to the ~50K protein derived from p105, p49 acts in synergy with p65 to stimulate the human immunodeficiency virus (HIV) enhancer in transiently transfected Jurkat cells. p49/p100 NF- κ B could therefore be important in the regulation of HIV and other κ B-containing genes.

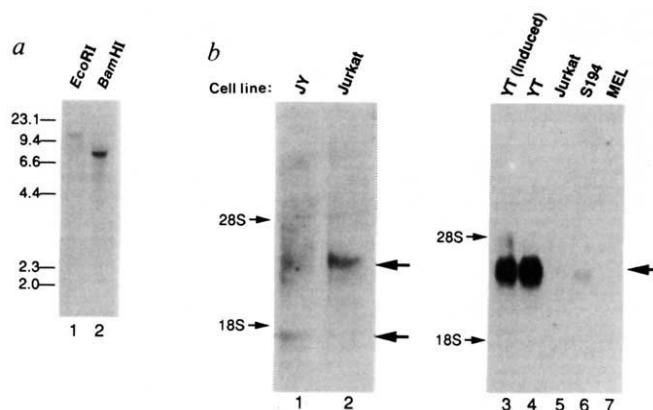


FIG. 1 DNA and RNA analysis of the p49 cDNA clone. *a*, Southern blot analysis of p49 on human genomic DNA (10 µg) cut with *Eco*RI (lane 1) or *Bam*HI (lane 2). The DNA probe used was a 1,069-base-pair *Bst*XI fragment of p49 which lacks both 5'-untranslated DNA and the repetitive region from the 3' end of the clone. Molecular size markers (in kb) are indicated on the left. *b*, Northern blot analysis of 10 µg poly(A)⁺ RNA from JY, an Epstein-Barr virus-transformed B-cell line (lane 1) (from J. Leiden), and the Jurkat T leukaemia line (lane 2) using the *Bst*XI fragment of p49 as a probe with GeneScreen Plus (DuPont). Another analysis was performed using 10 µg poly(A)⁺ RNA from TPA-stimulated and unstimulated YF T leukaemia cells (lanes 3 and 4); the Jurkat T leukaemia line (lane 5); S194, a murine B cell line (lane 6); and the murine erythroleukaemia (MEL), cell line (lane 7), on a nitrocellulose filter. Migration positions of ribosomal RNA markers are indicated (28S and 18S). Arrows denote specific hybridizable mRNA species of 1.9 kb (lanes 1, 2) and ~3.5 kb (lanes 1-7). For each blot, comparable amounts of actin RNA were detected in all lanes using a human β -actin probe.

Received 5 June; accepted 27 June 1991.

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ACKNOWLEDGEMENTS. We thank L. M. Fisher for teniposide. Ansamitocin was a donation from Takeda Chemical Industries of Japan. D.R. is supported by the SERC and this investigation received support from the UNDP/World Bank/WHO special programme for Research and Training in Tropical Diseases.

We have isolated cDNAs encoding the NF- κ B subunit proteins in order to characterize them and determine the mechanism of activation by NF- κ B. Trypsin-digested κ B binding proteins purified from bovine spleen were sequenced. Three peptides were identical to a DNA-binding subunit of human NF- κ B/KBF1 (ref. 3); another was 75% identical to a homologous peptide of p105. This peptide sequence suggested that an alternative NF- κ B protein could exist.

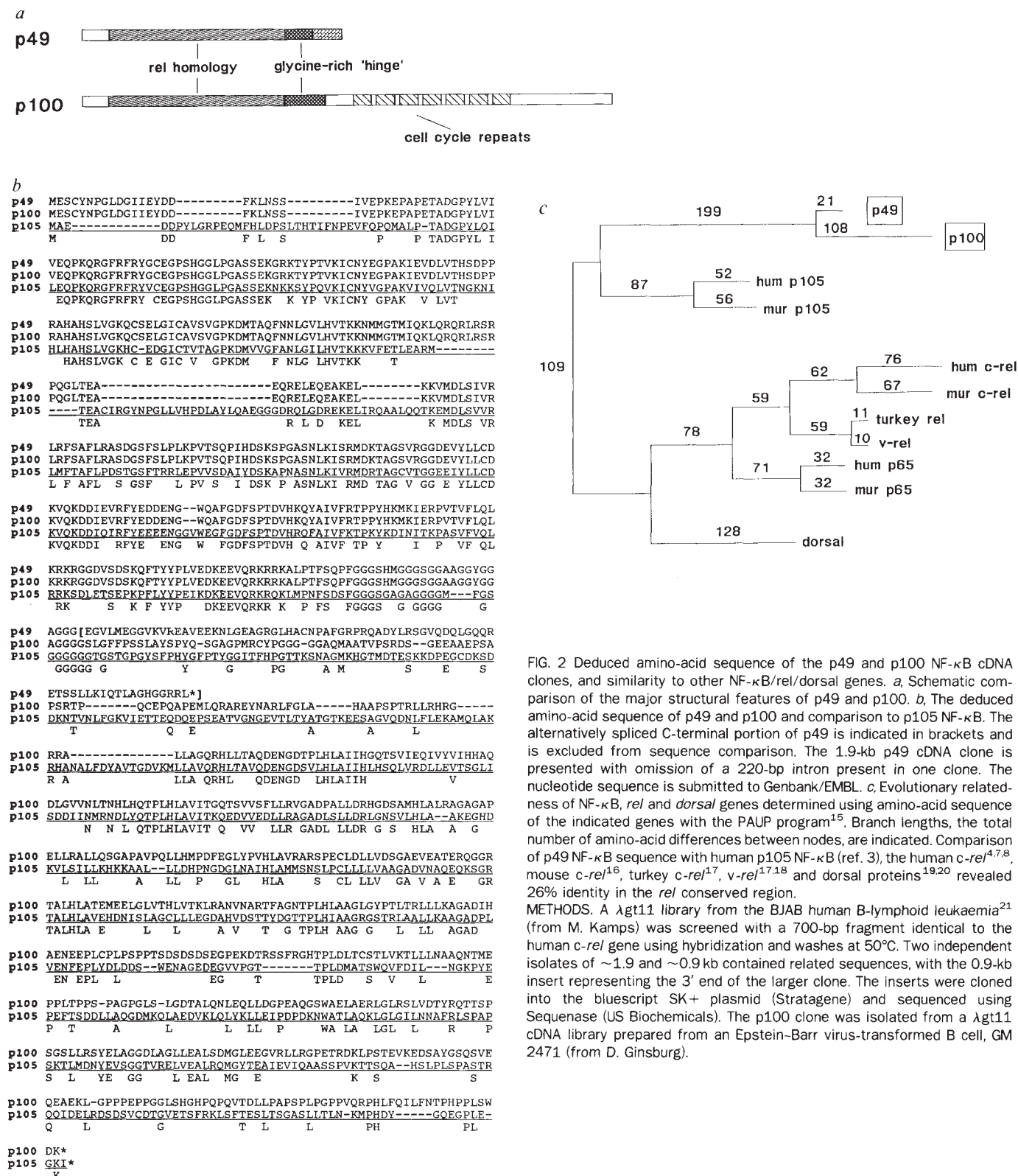


FIG. 2 Deduced amino-acid sequence of the p49 and p100 NF- κ B cDNA clones, and similarity to other NF- κ B/rel/dorsal genes. **a**, Schematic comparison of the major structural features of p49 and p100. **b**, The deduced amino-acid sequence of p49 and p100 and comparison to p105 NF- κ B. The alternatively spliced C-terminal portion of p49 is indicated in brackets and is excluded from sequence comparison. The 1.9-kb p49 cDNA clone is presented with omission of a 220-bp intron present in one clone. The nucleotide sequence is submitted to Genbank/EMBL. **c**, Evolutionary relatedness of NF- κ B, *rel* and *dorsal* genes determined using amino-acid sequence of the indicated genes with the PAUP program¹⁵. Branch lengths, the total number of amino-acid differences between nodes, are indicated. Comparison of p49 NF- κ B sequence with human p105 NF- κ B (ref. 3), the human *c-rel*^{4,7,8}, mouse *c-rel*¹⁶, turkey *c-rel*¹⁷, *v-rel*^{17,18} and dorsal proteins^{19,20} revealed 26% identity in the *rel* conserved region.

METHODS. A λ gt11 library from the BJAB human B-lymphoid leukaemia²¹ (from M. Kamps) was screened with a 700-bp fragment identical to the human *c-rel* gene using hybridization and washes at 50°C. Two independent isolates of ~1.9 and ~0.9 kb contained related sequences, with the 0.9-kb insert representing the 3' end of the larger clone. The inserts were cloned into the bluescript SK+ plasmid (Stratagene) and sequenced using Sequenase (US Biochemicals). The p100 clone was isolated from a λ gt11 cDNA library prepared from an Epstein-Barr virus-transformed B cell, GM 2471 (from D. Ginsburg).

(Fig. 1b), demonstrating that there are several species of p49 whose abundance varies among different cell types. For example, the larger 3.5 kb RNA was found in all the cells we examined and was predominant in YT T leukaemia cells, but both transcripts were present equally in the JY B-cell line (Fig. 1b, lanes 1 and 3).

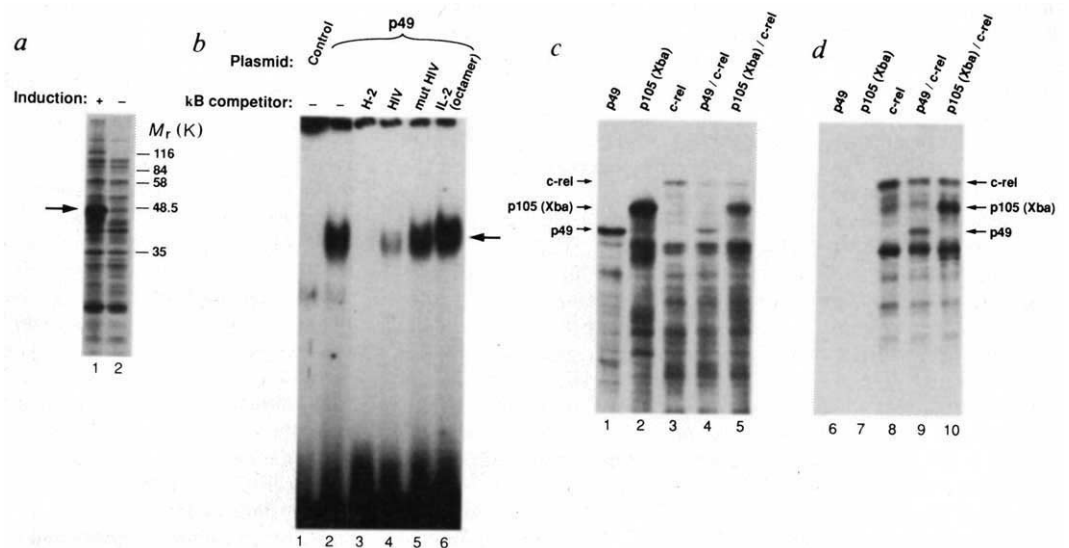
To characterize the larger messenger RNA species, we isolated and sequenced additional cDNA clones. The clone for the shorter transcript contained a 1,341-base pair (bp) open reading frame, encoding a protein of predicted $M_r \sim 49,100$ (Fig. 2a, b); the longer clone encoded a protein of predicted $M_r \sim 100,634$. These sequences were identical through amino acid 374, into the glycine-rich putative hinge region, after which they diverged. The amino-acid sequence in the common N-terminal region was similar (26% identity) to that in p105 NF- κ B, and in the *dorsal*

and *rel* proteins (Fig. 2a), the greatest similarity being between p49/p100 and p105 (60% identity). One subregion contained conserved cysteine and histidine residues which do not conform to a classic zinc-finger motif. Interestingly, κ B-binding activity depends on zinc⁹, and, like the *tat-1* gene of HIV, may form an alternative structure which participates in dimerization or nucleic acid binding. The longer transcript contains repeated sequences in the C-terminal region which have homology with motifs in p105 and proteins encoded by cell cycle genes³⁻⁶. In this family, p100 NF- κ B is most closely related to p105 NF- κ B (41% identity), and p65 to *c-rel* protein (50% identity) (Fig. 2c).

To investigate the DNA-binding activity of p49, we used a prokaryotic expression system and an electrophoretic mobility shift assay. The predominant product of ~ 49 K (Fig. 3a, lane 1) displayed specific κ B binding activity (Fig. 3b, lane 2 versus

FIG. 3 κ B binding and heterodimerization of the p49 cDNA clone. **a**, SDS-PAGE of recombinant p49 visualized by Coomassie brilliant blue staining in an induced (+) or uninduced ($-$) *Escherichia coli* strain. The inducible protein band is indicated by an arrow. **b**, Electrophoretic mobility shift assays of bacterially expressed control (lane 1) or p49 protein (lanes 2-6). The assay was performed using a double-stranded ³²P-labelled oligonucleotide probe containing the H-2K^b enhancer κ B site (0.1 ng). Unlabelled κ B sites (100 ng) from the indicated enhancers were used for the competition reactions. Arrow denotes specific inducible complex. **c**, SDS-PAGE (lanes 1-5) and **d**, immunoprecipitation (lanes 6-10) of cDNAs translated *in vitro* using wheat-germ lysates. Immunoprecipitations were performed with a polyclonal antiserum raised against *v-rel* protein. [³⁵S]methionine-labelled protein extracts were resolved on a 10% SDS-polyacrylamide gel and visualized by autoradiography. Arrows indicate full-length p49, p105 (XbaI) and *c-rel* protein.

METHODS. p49 was expressed in *E. coli* using the pET system in the pLysE

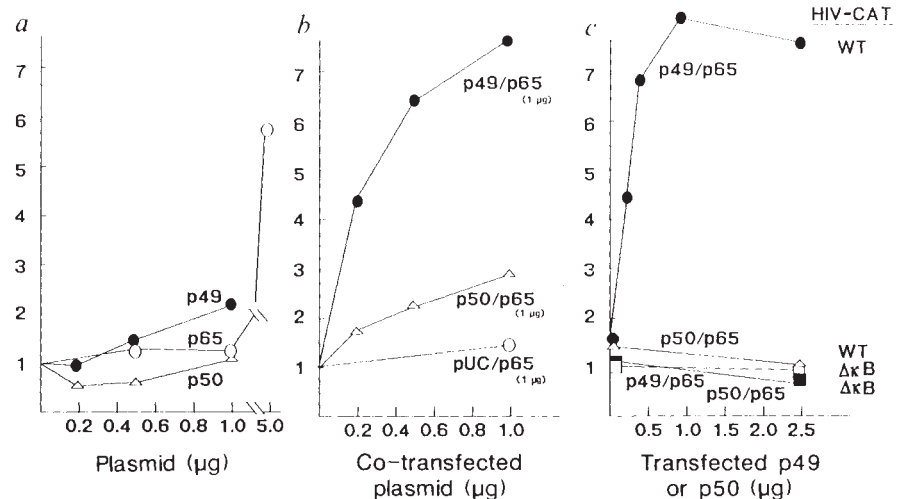


strain²². Protein extracts were prepared as described¹⁰, except buffer A contained 0.05% Nonidet-P40, and glycerol was added to a concentration of 20% (v/v); electrophoretic mobility shift assay was performed by standard methods^{23,24} using $\sim 0.4 \mu$ g protein extract. The control extract was prepared from a p49 deletion mutant lacking the first 317 N-terminal amino acids.

FIG. 4 Transcriptional activation and synergistic function of p49/p65 *in vivo*. Jurkat cells were transfected with a reporter gene containing 4 κ B sites linked to CAT (ref. 23) and eukaryotic expression vectors encoding p49, p105 (*RsaI*) or 'p50' and p65 alone (**a**) or in the indicated combinations with a suboptimal constant amount of p65 (**b**). Jurkat cells were also transfected with HIV-CAT or its κ B mutant²⁴ with the indicated combinations and amounts of p49 or p50 (p105 (*RsaI*)) expression plasmids (**c**) and a constant amount of p65 (1 or 2.5 μ g). pUC 9 plasmid was added to make a total amount of 20 μ g DNA per transfection. In separate experiments, no additional transactivation was observed with 5 μ g of p49 or p105(*RsaI*) alone in **a**, and similar results were observed with p105(*RsaI*) and p105(*XbaI*) (data not shown). WT, Wild type.

METHODS. Full-length p105 cDNA was isolated from the GM 2471 human B-cell λ gt11 library.

The *RsaI* truncation was generated by insertion of a stop codon at the position corresponding to amino acid 402, followed by an *XhoI* restriction site inserted by site-directed mutagenesis²⁴, which allowed deletion of the 3' end of the p105 *RsaI*-truncated gene. Mouse p65 cDNA was obtained by PCR with 702 B-cell cDNA using primers from the sequence²⁵ correspond-



ing to amino acids 1-546. All cDNAs had a *HindIII* restriction site and a consensus Kozak sequence (AAGCTTCACCATG). The *HindIII* site allowed subcloning into a Rous sarcoma virus β globin expression vector²⁶ in which the β globin coding sequence had been removed by digestion with *HindIII* and *BglII*.

lane 1) and could compete with the H-2 and HIV κ B sites, but not with a single-base-pair mutant of HIV or with an unrelated interleukin-2 octamer site (Fig. 3b, lanes 3 and 4 versus 5 and 6). We tested the interaction of p49 with other *rel* proteins by immunoprecipitation in a wheat-germ co-translation system: like p105 (*XbaI*), p49 also associated with c-*rel* protein (Fig. 3d).

To determine whether p49 interacts with other NF- κ B/*rel* proteins to stimulate transcription, eukaryotic expression vectors were transfected into Jurkat cells. Transfection of p49 alone stimulates κ B enhancer activity slightly; p65 at higher concentrations significantly increases κ B-dependent transcription (Fig. 4a). Transfection of small amounts ($\leq 1 \mu\text{g}$) of either p49 or p65 causes minimal stimulation, but when co-transfected with each other, they act together to stimulate a κ B reporter plasmid. This stimulation was more effective than the combination of 'p50' (p105(*RsaI*)) and p65 (Fig. 4b), suggesting that p49 was more effective in cooperating with p65. When analysed with the HIV-chloramphenicol acetyl transferase (HIV-CAT) reporter, the p49/p65 combination, but not p50 (p105(*RsaI*))/p65, stimulated HIV-CAT activity, an effect that required an intact κ B regulatory element (Fig. 4c). Truncated p100 (48.5K form) gave a comparable stimulation, in contrast to full-length p100, which was inactive (data not shown), suggesting that differences in the *rel* conserved domain mediate this effect.

These findings demonstrate that κ B-dependent transcription is regulated by the p49/100 gene products. The p65 and *rel* proteins are putative transcriptional activation subunits with intrinsic DNA-binding activity which associate with another DNA-binding subunit, previously thought to be a single gene product derived from p105. These findings suggest that p49/100 is an alternative DNA-binding subunit of NF- κ B which acts with p65 to activate κ B-dependent transcription. Many proteins can bind to κ B-related sites, some of which are not NF- κ B/*rel*-related (refs 10–12; and B. Adams, K. Leung, E. Hanley and G. J. N., manuscript submitted). Although some κ B-binding proteins have been characterized^{13,14}, their identification is equivocal as they have related antigenic epitopes in their amino-terminal region. As with p49/100, the identification of these cDNAs will allow them to be analysed further. The interaction of p49/100 with other proteins and its other possible modes of regulation may provide additional mechanisms to regulate the transcription of HIV and different κ B-containing genes. $\square$

Received 8 May; accepted 8 July 1991.

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ACKNOWLEDGEMENTS. This work resulted from an equal contribution by the first two authors. We thank D. Gschwend for typing assistance; E. Hanley for technical expertise; B. Adams and B.-y. Wu for northern blot filters; N. Rice for c-*rel* expression vectors and antibodies; A. Israel for analysing and sharing peptide sequence before publication; G. E. Griffin and D. Ginsburg for advice, discussion and help with computer graphics; and D. Siemieniuk for help with computer analyses. This work was supported in part by a DFG Fellowship (R.M.S.), the British Medical Research Council's AIDS Directed Programme (C.S.D.), and a grant from the National Institutes of Health (G.J.N.). C.S.D. is also affiliated with St George's Hospital Medical School in London.

A protein-tyrosine phosphatase with sequence similarity to the SH2 domain of the protein-tyrosine kinases

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THE phosphorylation of proteins at tyrosine residues is critical in cellular signal transduction, neoplastic transformation and control of the mitotic cycle¹. These mechanisms are regulated by the activities of both protein-tyrosine kinases (PTKs) and protein-tyrosine phosphatases (PTPases)². As in the PTKs, there are two classes of PTPases: membrane associated, receptor-like enzymes^{3–5} and soluble proteins^{3,6,7}. Here we report the isolation of a complementary DNA clone encoding a new form of soluble PTPase, PTP1C. The enzyme possesses a large noncatalytic region at the N terminus which unexpectedly contains two adjacent copies of the Src homology region 2 (the SH2 domain) found in various nonreceptor PTKs⁸ and other cytoplasmic signalling proteins^{9–11}. As with other SH2 sequences, the SH2 domains of PTP1C formed high-affinity complexes with the activated epidermal growth factor receptor and other phosphotyrosine-containing proteins. These results suggest that the SH2 regions in PTP1C may interact with other cellular components to modulate its own phosphatase activity against interacting substrates. PTPase activity may thus directly link growth factor receptors and other signalling proteins through protein-tyrosine phosphorylation.

Screening of a human breast carcinoma (ZR-75-1) cDNA library with a LAR cDNA probe⁴ under conditions of low stringency⁵ yielded many positive phage clones. Southern blotting showed that four of these clones hybridized to each other under conditions of high stringency. Two clones with large cDNA inserts, PTP1C4 (2.3 kilobases (kb)) and PTP1C2 (2.1 kb), were chosen for further characterization. The sequence of the largest cDNA, PTP1C4, had an open reading frame potentially encoding a polypeptide of 609 amino acids (Fig. 1). The sequence of the second largest cDNA clone, PTP1C2, was identical to that of PTP1C4 but started at nucleotide 249 of PTP1C4. Northern blot analysis of RNA from ZR-75-1 cells¹² with the PTP1C cDNA as a probe detected a single transcript of about 2.4 kb. The size of this transcript agrees with the size of PTP1C4 cDNA insert.

To demonstrate that the cDNA PTP1C clone encodes an active PTPase, the PTP1C2 cDNA insert was subcloned into the *Bam*HI site of the expression vector pET-3c (ref. 13) to generate the plasmid pT7-PTP1C. *Escherichia coli* cells transformed with this plasmid were induced with isopropylthiogalactoside (IPTG) and cell lysates were assayed for PTPase activity. Although *E. coli* extracts carrying a control plasmid have no detectable PTPase activity, cells transformed with pT7-PTP1C have very high amounts of PTPase activity (Fig. 2). These results indicate that the deduced protein sequence in Fig. 1 encodes a functional PTPase.

Computer analysis of the amino-acid sequences of PTP1C did not find a sequence with the characteristics of a signal peptide in the N-terminal region. Neither was a characteristic transmembrane sequence found in the entire protein sequence. Accordingly, it is concluded that PTP1C is a cytosolic soluble PTPase. Analysis of the primary structure of PTP1C showed

that the PTPase domain, a 246 amino-acid core sequence⁵, is located in the middle of the protein (Fig. 3a). In comparison with mammalian soluble PTPases so far reported, PTP1C has a smaller noncatalytic C-terminal domain (93 amino acids) which lacks the hydrophobic sequence found in the other soluble PTPases⁷. Unlike the other mammalian soluble PTPases, PTP1C has a large noncatalytic N-terminal domain (270 amino acids). In this noncatalytic domain two adjacent segments roughly 90 amino acids long, one starting at Trp6 (N) and the other at Trp112 (C), have good sequence similarity to the SH2 domain. This domain was initially identified at the N-terminal, non-catalytic region of various Src-like PTKs⁸ and was subsequently found in a number of cytoplasmic signalling proteins, including

phospholipase C- γ 1 (PLC- γ 1), GTPase-activating protein (GAP), the transforming protein encoded by *crk* (refs 9–11) and the actin-binding protein, tensin¹⁴. The overall sequence identity of PTP1C to the corresponding SH2 sequences is more than 45% (Fig. 3b).

The SH2 domain of GAP, PLC- γ 1, p85 and Src mediate the physical association of these proteins with activated epidermal growth factor receptor (EGF-R), platelet-derived growth factor-receptor (PDGF-R) and other tyrosine-phosphorylated proteins^{10,15–17}. The conserved N-terminal SH2 domain is also essential for the transforming activity of oncogenes⁸. The SH2 domain of tensin binds specifically to a number of phosphotyrosine-containing proteins from v-src-transformed cells¹⁴.

FIG. 1 Nucleotide and deduced amino-acid sequence of the PTP1C cDNA. The nucleotides are numbered from the 5' end of the 2.3-kb cDNA clone PTP1C4. The putative polyadenylation signal AATAAA (nucleotide 2,242) is overlined. The two segments of the SH2 sequences are underlined. The first ATG codon in the open reading frame at position 256 may serve as the translation initiation codon. The open reading frame terminates at nucleotide 2,082 with a TGA stop codon. The two vertical arrows demarcate the 246-residue PTPase domain.

METHODS. A commercial cDNA library (Clontech) made from the human breast carcinoma cell line ZR-75-1 (ref. 12) was screened for PTPase clones. The probe used in screening in the library was a 1.5-kb *EcoRI-SalI* DNA fragment spanning the two PTPase domains of human LAR cDNA (ref. 4). The probe was labelled with [³²P]-dNTP using a random labelling kit (Pharmacia). Hybridization and washing conditions (low stringency) were as described previously⁵. cDNA inserts were subcloned into pBluescript KS vectors (Stratagene) and both strands of cDNA completely sequenced using the DNA sequencing kit (Pharmacia).

CAAGAAGACGGGGATTGAGGAGGCCCTCAGGCGCCTTTGTCTACCTGCGGCAGCCGTACTATGCCACGAGGGTGAATGCGGCTGA	84
CATTGAGAACCGAGTGTGGAACTGAACAAGAAGCAGGAGTCCGAGGAGGAAGTGGCTGATTACTGAGCGGTTCTCTCCACCT	168
GGCTTGGGCCACTGTGCACAGCTGTGCCGCTGGCTCAGCCCCCCCCCTGCGGCCCTCCGCCGTGGCTTCCCCCTCCCTACAGA	252
GAGATGTCTGCCGTGGGTGGTTTACCAGAGACCTCAGTGGGTGGATGCAGAGACCTGTCAAGGGCCGAGGTGCCACGGT	336
METLeuSerArgGlyTrpPheHisArgAspLeuSerGlyLeuAspAlaGluThrLeuLeuLysGlyArgGlyValHisGly	27
AGCTTCCTGGCTCGGCCAGTCGCAAGAACAGGGGTGACTTCTCGCTCTCCGTACGGGTGGGGATCAGGTGACCCATATTCGG	420
SerPheLeuAlaArgProSerArgLysAsnGlnGlyAspPheSerLeuSerValArgValGlyAspGlnValThrHisIleArg	55
ATCCAGAACTCAGGGGATTTCTATGACCTGTATGGAGGGGAGAAGTTTGGCACTCTGACAGACGTGGTGGAGTACTACACTCAG	504
IleGlnAsnSerGlyAspPheTyrAspLeuTyrGlyGlyGluLysPheAlaThrLeuThrGluLeuValGluTyrTyrThrGln	83
CAGCAGGGTGTCTGCAGGACCGGCAGCGCACCATCATCCACCTCAAGTACCCGCTGAAGTGTCTCCGATCCCACTAGTGAGAGG	588
GlnGlnGlyValLeuGlnAspArgAspGlyThrIleIleHisLeuLysTyrProLeuAsnCysSerAspProThrSerGluArg	111
TGGTACCATGGCCATGTCTGCGCGGCAGGCAGAGACGTGTGACAGGCCAAGGGCAGCCCTGGAGCTTTCTTGTGCGTGAG	672
TrpTyrHisGlyHisMetSerGlyGlyGlnAlaGluThrLeuLeuGlnAlaLysGlyGluProTrpThrPheLeuValArgGlu	139
AGCCTCAGCCAGCCTGGAGACTTCGTGCTTTCTGTCTCAGTACAGCCCAAGGCTGGCCAGGCTCCCCGCTCAGGGTCACC	756
SerLeuSerGlnProGlyAspPheValLeuSerValLeuSerAspGlnProLysAlaGlyProGlySerProLeuArgValThr	167
CACATCAAGTTCATGTGCGAGGGTGGACGCTACACAGTGGGTGGTTTGGAGACCTTCGACAGCCTCACGGACCTGGTAGAGCAT	840
HisIleLysValMetCysGluGlyGlyArgTyrThrValGlyGlyLeuGluThrPheAspSerLeuThrAspLeuValGluHis	195
TTCAAGAAGACGGGGATTGAGGAGGCCCTCAGGCGCCTTTGTCTACCTGCGGCAGCCGTACTATGCCACGAGGGTGAATGCGGCT	924
PheLysLysThrGlyIleGluGluAlaSerGlyAlaPheValTyrLeuArgGlnProTyrTyrAlaThrArgValAsnAlaAla	223
GACATTGAGAACCGAGTGTGGAACTGAACAAGAAGCAGGAGTCCGAGGATACAGCCCAAGGCTGGCTTCTGGGAGGAGTTTGG	1008
AspIleGluAsnArgValLeuGluLeuAsnLysLysGlnGluSerGluAspThrAlaLysAlaGlyPheTrpGluGluPheGlu	251
AGTTTGCAGAACGAGGAGGTGAAGAACTTGCACACGCGCTGGAAGGGCAGCGGCCAGAGAACAAGGGCAAGAACCGCTACAAG	1092
SerLeuLysLysGlnGluValLysAsnLeuHisGlnArgLeuGluGlyGlnArgProGluAsnLysGlyLysAsnArgTyrLys	279
AACATTCTCCCTTTGACCACAGCCGAGTGATCTGCAGGGACGGGACAGTAACATCCCCGGGTCCGACTACATCAATGCCAAC	1176
AsnIleLeuProPheAspHisSerArgValIleLeuGlnGlyArgAspSerAsnIleProGlySerAspTyrIleAsnAlaAsn	307
TACATCAAGAACGAGCTGCTAGGCCCTGATGAGAACGCTAAGACCTACATCGCCAGCCAGGGCTGTCTGGAGGCCACGGTCAAT	1260
TyrIleLysAsnGlnLeuLeuGlyProAspGluAsnAlaLysThrTyrIleAlaSerGlnGlyCysLeuGluAlaThrValAsn	335
GACTTCTGGCAGATGGCGTGGCAGGAGAACAGCCGTGTCATCGTCATGACCACCCGAGAGGTGGAGAAAGGCCGGAACAAATGC	1344
AspPheTrpGlnMetAlaTrpGlnGluAsnSerArgValIleValMetThrThrArgGluValGluLysGlyArgAsnLysCys	363
GTCCCATCTGGCCGAGGTGGGCATGCAGCGTGCTTATGGGCCCTACTCTGTGACCAACTGCGGGGAGCATGACACAAACCGAA	1428
ValProTyrTrpProGluValGlyMetGlnArgAlaTyrGlyProTyrSerValThrAsnCysGlyGluHisAspThrThrGlu	391
TACAAACTCCGTACCTTACAGGTCTCCCCGCTGGACAATGGAGACCTGATTCCGGAGATCTGGCATTACCAGTACCTGAGCTGG	1512
TyrLysLeuArgThrLeuGlnValSerProLeuAspAsnGlyAspLeuIleArgGluIleTrpHisTyrGlnTyrLeuSerTrp	419
CCCGACCATGGGGTCCCCAGTGGAGCTGGGGTGTCTCAGCTTCCTGGACAGATCAACAGCGGCAGGAAAGTGTGCTCAG	1596
ProAspHisGlyValProSerGluProGlyGlyValLeuSerPheLeuAspGlnIleAsnGlnArgGlnGluSerLeuProHis	447
GCAGGGCCCATCATCGTGCATGCAGCGCCGCGCATCGGCCGACAGGCACCATCATGTCTCATGACATGCTCATGGAGAACATC	1680
AlaGlyProIleIleValHisCysSerAlaGlyIleGlyArgThrGlyThrIleIleValIleAspMetLeuMetGluAsnIle	475
TCCACCAAGGGCCTGGACTGTGACATTGACATCCAGAAGACCATCCAGATGGTGGCGGCGCAGCGCTCGGGCATGGTGACAGC	1764
SerThrLysGlyLeuAspCysAspIleAspIleGlnLysThrIleGlnMetValArgAlaGlnArgSerGlyMetValGlnThr	503
GAGGCGCAGTACAAGTTCATCTACGTGGCCATCGCCAGTTTCATTGAAACCACTAAGAAGAAGCTGGAGGTCTGCAGTCGAG	1848
GluAlaGlnTyrLysPheIleTyrValAlaIleAlaGlnPheIleGluThrThrLysLysLysLeuGluValLeuGlnSerGln	531
AAGGGCCAGGAGTCCGAGTACGGGAACATCACTATCCCCAGCCATGAAGAATGCCATGCCAAGGCCTCCCGCACCTCGTCC	1932
LysGlnGlnGluSerGluTyrGlyAsnIleThrTyrProLeuAlaHisAlaLysAlaSerAspThrSerSerTrp	559
AAACACAAGGAGGTGTGTATGAGAACCTGCACACTAAGAACAGAGGGAGGAGAAAGTGAAGAAGCAGCGGTACGACAGACAAG	2016
LysHisLysGluAspValTyrGluAsnLeuHisThrLysAsnLysArgGluGluLysValLysLysGlnArgSerAlaAspLys	587
GAGAAGAGCAAGGTTCCCTCAAGAGGAAGTGAGCGGTGTCTCAGGTGGCCATGCCTCAGCCCTGACCTGTGGAAGCATT	2100
GluLysSerLysValProSerArgGlySerGluArgCysCysProGlnValAlaMetProGlnPro	609
TCCGATGGACAGACTCAACCTGAACCTAGGAGTGCCCATCTTTTGAATTAATGGCTGCATCCCCCCCCACCTCTCCC	2184
TGACCTGTATATAGCCAGCCAGGCCCCAGGCGAGGGCCAAACCTTCTCTCTTGTAAATAAAGCCCTGGGATCACTGAAAAAA	2268
AAAAAAAA	2276

These reports prompted us to explore whether the SH2 domain from PTP1C would also bind phosphotyrosine-containing proteins *in vitro*.

To examine the binding ability of the SH2 sequences from PTP1C, a restriction fragment containing the two adjacent SH2 domains was excised from PTP1C, cloned into a bacterial vector (pMAL-c) and expressed in *E. coli* as a fusion to a maltose-binding protein (MBP) leader segment¹⁸. The SH2-containing fusion protein was immobilized on maltose-conjugated resin. Lysates of A431 cells¹⁹ were phosphorylated *in vitro* by EGF and ATP²⁰ and applied to the resin for binding. The bound proteins were eluted by a maltose-containing solution and analysed by protein immunoblotting with a monoclonal antibody specific for phosphotyrosine. A number of phosphotyrosine-containing proteins bound specifically to the MBP-

SH2 fusion protein but not to the leader MBP alone (Fig. 4). The major band of tyrosine-phosphorylated proteins bound to the SH2 domains was a species of relative molecular mass of 180,000 (M_r , 180K). Other bands, roughly 110, 70 and 58-60K were also detected by the anti-phosphotyrosine antibody. In duplicate samples, the 180K phosphotyrosine protein consistently reacted with anti-EGF-R antibody, suggesting that this protein was EGF-R. When A431 cells were stimulated with EGF *in vivo*, the 180K phosphorylated EGF-R protein remained the predominant band whereas the minor bands detected under *in vitro* stimulation conditions were not observed, or were at substantially reduced levels (data not shown). This is consistent with the fact that stimulation *in vitro* invariably produced more protein-tyrosine phosphorylation. This extensive protein-tyrosine phosphorylation *in vitro* may be responsible for binding

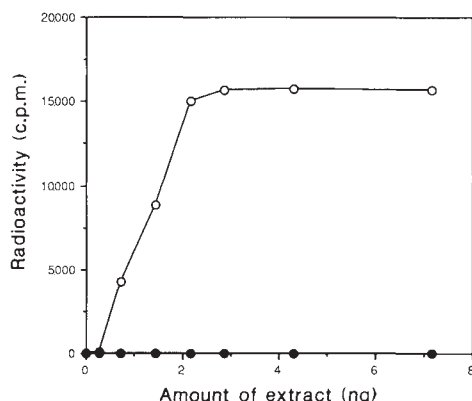


FIG. 2 PTPase activity of recombinant PTP1C expressed in *E. coli*. Dephosphorylation of the synthetic peptide, Raytide, was assayed for the recombinant PTP1C. The y axis represents the released radioactivity from the substrate. Extracts from bacteria carrying pET-3c and pT7-PTP1C are indicated by closed and open circles, respectively.

METHODS. The cDNA clone PTP1C2, was used for expression of the protein in *E. coli*. The 5' end of this cDNA clone is nucleotide 249 of the cDNA sequence, and therefore has only seven additional nucleotides upstream from the start codon ATG of the PTP1C coding region. The *EcoRI* fragment (2 kb) of the clone was inserted in frame into the *Bam*HI site of the pET-3c expression vector¹³ after filling in the ends with Klenow DNA polymerase. The resulting construct encodes for 17 additional amino acids derived from the vector and the noncoding sequence of the cDNA clone upstream of the putative start codon. The construct was transformed into *E. coli* BL21 (DE3), which carries the T7 polymerase gene under control of the *lacUV5* promoter. The expression of PTPase in the transformed cells was induced by adding 25 μ M IPTG into the culture medium. To assay PTPase activity, Raytide (Oncogene Science) was labelled on tyrosine residues using γ -³²P-ATP and p43^{v-abl} tyrosine kinase (Oncogene Science) and used as a PTPase substrate⁵. *E. coli* extracts were prepared and assayed for PTPase activity as described by Krueger *et al.*⁵.



FIG. 3 Sequence similarities between the two segments of PTP1C and other SH2 sequences. *a*, A schematic representation of PTP1C structure. The locations of the N-terminal noncatalytic segment, the PTPase domain, and the C-terminal segment are indicated. The hatched boxes in the N-terminal segment represent the locations of the two SH2 domains. *b*, Amino-acid alignment of the SH2 domains taken from the nonreceptor PTKs. Boxed

residues are identical to PTP1C. Gaps imposed to maximize alignment are indicated by dots. Number in parentheses is the first amino acid of the sequence shown. The amino-acid sequences of the proteins encoded by the following are presented: *Isk* (ref. 21), *hck* (ref. 22), *fyn* (ref. 23), *Dsrc* (ref. 24), *tk1* (ref. 25), *yes* (ref. 26), *fert* (ref. 27), *src* (ref. 28), *abl* (ref. 29), *Dsrc28* (ref. 30).

of other species to the SH2 domains (Fig. 4). Because the SH2 fusion protein contained an additional stretch of 29 amino acids (see Fig. 4 legend), we cannot exclude the possibility, but we think it unlikely, that this stretch bound those phosphotyrosine-containing proteins.

In general, the function of PTPases is thought to counter-balance the action of PTKs in order to regulate the phosphotyrosine content of proteins involved in cellular signal transduction and other functions. But the precise mechanism by which the PTKs and the PTPases interact together to achieve a desired phosphorylation level in cells for regulation of signal transduction is unknown. Recently, the function of the conserved SH2 domain in PTKs and related signalling proteins has been elucidated. Such domains mediate the formation of heteromeric protein complexes which further control the activation of signalling transduction pathways by PTKs¹⁵. The presence of the SH2 domains in PTP1C suggests that PTP1C, perhaps as one member of a family of SH2-containing PTPases, uses a similar mechanism to control the activity of receptor PTKs and hence phosphorylation. Although the endogenous interacting proteins bound by the SH2 domain of PTP1C *in vivo* are yet to be identified, it is possible that the SH2 sequences in PTP1C

direct the enzyme to specific substrates for interaction, modulation of activity and selective dephosphorylation of substrates in response to a change in the cellular environment. Thus, the finding of the SH2 domain in PTP1C indicates the direct linkage of PTPase to the growth factor-receptors and other signalling proteins for the regulation of signal transduction and other related functions through phosphorylation and dephosphorylation of tyrosine residues. □

Received 24 June; accepted 2 August 1991.

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ACKNOWLEDGEMENTS. We thank D. Banville for advice, H. Saito for sending LAR probes, M. O'Connor and J. F. Morin for help and D. Thomas and S. Siliaty for critical comments on the manuscript.

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FIG. 4 Binding of phosphotyrosine-containing proteins to the SH2 domains of PTP1C. A recombinant fusion protein (about 10 µg) containing the two SH2 domain of PTP1C and the MBP (ref. 18) was synthesized in *E. coli* and immobilized on a column of maltose-conjugated resin (0.5 ml) by using a New England Biolabs Kit. The immobilization column was washed with binding buffer (10 mM phosphate, 30 mM NaCl, 1 mM azide, 1 mM EGTA, 5 mM MgCl₂, 0.5 mM orthovanadate and protease inhibitors as in PLCLB buffer¹⁰ (50 mM Hepes, pH 7.0, 150 mM NaCl, 10% glycerol, 1% Triton X-100, 1.5 mM MgCl₂, 1 mM EGTA, 100 mM NaF, 10 mM NaPP_i, 1 mM Na₃VO₄, 1 mM phenylmethylsulphonyl fluoride, aprotinin and leupeptin each at 10 µg ml⁻¹). About 2 × 10⁷ A431 cells were lysed in 1 ml PLCLB and phosphorylated as described by Otsu *et al.*²⁰, except that cell lysates were first stimulated with 100 nM EGF *in vitro* for 15 min at 4 °C before adding ATP. Cell lysates (200 µl) were diluted with four volumes of the binding buffer and passed through the column four times by reapplying the flow-through to the column. The column was washed two times with five column volumes of binding buffer. The bound material was eluted from the column with an elution buffer (binding buffer plus 10 mM maltose and 0.5 M NaCl). One third of the eluted materials were resolved on 7% SDS-polyacrylamide gels and analysed by protein immunoblotting with a monoclonal antibody specific for phosphotyrosine (UBI) or a polyclonal antibody reactive to human EGF-R (a gift from S. Grothe). Bars are molecular mass standards. Arrow indicates the position of EGF-R.

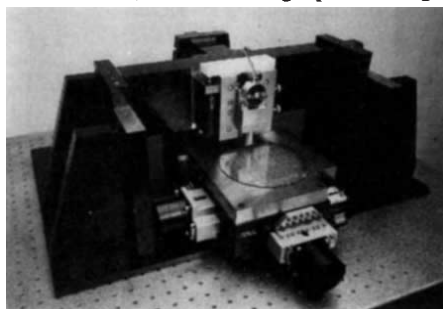
METHOD. To express the SH2 domain, cDNA clone PTP1C2 was excised by *EcoRI* (immediately before the start codon ATG) and *SmaI* (nucleotide 1,152) digestion. The excised fragment, consisting of the entire noncatalytic N terminus and the first 29 residues of the catalytic domain, was cloned into the expression vector pMAL-c (ref. 18) at the *EcoRI* and the *HindIII* site which had been filled in for blunt ligation. The expressed fusion protein contains a leader MBP of 42K. Expression and immobilization of the fusion protein were done according to the manufacturer's protocol.

ACS in the Big Apple

Next week's American Chemical Society meeting to be held in New York City will feature an automated peptide synthesizer based on a proprietary dry-activation Fmoc chemistry, a mass analyser for molecular weight determination from picomole amounts, and an NMR software module.

THE new Cytostir bioreactors with built-in stirring blades from Kontes are designed to keep microcarriers suspended during cell culture (*Reader Service No. 100*). The bioreactors are available in seven sizes: 100, 250, 500, 1,000, 3,000, 6,000 and 8,000 ml. All wetted surfaces are made of borosilicate glass or PTFE. The large PTFE stirring blades keep microcarriers in suspension at low stirring speeds, Kontes says. As a further measure, the blades are height-adjustable. A dome in the centre of the flask's base is also designed to prevent microcarriers from accumulating. Other design features include extra thick glass at side-arm stress points for added durability and an anti-drip pour lip on the side arms of bioreactors that are one litre or larger. All components of the Cytostir bioreactors are steam autoclavable. Prices range from \$91.57 to \$311.79 (US), depending on size. Kimble/Kontes will be in booth 804.

With the new large-sample scanning probe microscope (SPM) from Digital Instruments samples as large as 200 mm in diameter can be accommodated (*Reader Service No. 101*). The large-sample SPM images surfaces with three-dimensional resolution using either scanning tunnelling microscopy or atomic force microscopy. According to Digital Instruments, the X-Y stage positioning,



Digital Instruments' SPM for samples a little on the large side.

which can be controlled manually or automatically, is accurate to within ± 10 microns. The area in which the probe can be translated is 150×150 mm. Once the probe is positioned, it is possible to scan areas from a few nanometres up to 130-micron square. An integral optical microscope with one-micron resolution provides sample survey and precise tip alignment, the company says. As a further measure, the X-Y translation

stage can be removed to accommodate even larger samples. Drop by Digital Instruments' booth, number 737.

The new 1020 LC Plus Controller from Perkin-Elmer is designed to provide an 'intelligent' alternative to conventional controllers, offering single-point control of the LC system and data processing and chromatography monitoring at the local level (*Reader Service No. 102*). Occupying only 12 inches of bench space, the 1020 LC controller features a built-in, nine-inch CRT (allowing analysts to view and manipulate chromatograms in real time), drop-down menus and long-term data storage. The controller also provides 'hot status' information about running conditions of the LC system, including details of the flow rate, pressure readings, solvent percentages, run times, method number and injection number. In addition, users can view one or two real-time plots and change attenuation and time at will. Peruse Perkin-Elmer's booth, number 1011.

Time-saving tools

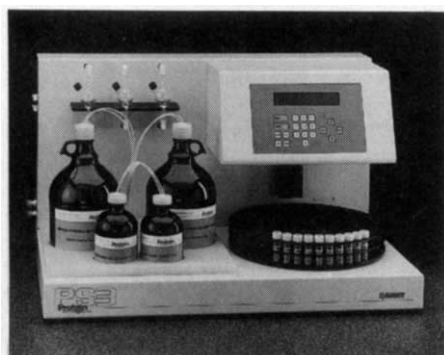
Bio-Rad's Model 491 preparative electrophoresis cell — the Prep Cell — is designed for the purification of specific proteins from complex mixtures by continuous-elution electrophoresis (*Reader Service No. 103*). The Prep Cell should be useful to those researchers who are purifying proteins for use in sequencing, antibody production, enzyme kinetic studies, or any *in vitro* and *in vivo* study where pure protein is required. Using SDS or native-PAGE, the Prep Cell can be used to isolate specific components from complex mixtures containing 100 ng to more than 50 mg of total protein. During a run, which typically takes 4–8 hours, molecules are electrophoresed through a cylindrical sieving gel matrix. Individual protein bands migrate off the bottom of the gel where they pass directly into the elution chamber for collection. A peristaltic pump drives separated proteins through a UV monitor to a fraction collector. As individual molecules are purified, they are immediately available for assay and characterization, Bio-Rad says. The Prep Cell, when used in combination with Bio-Rad's isoelectric focusing cell, provides users with a preparative two-dimensional electrophoresis system that can be used to purify proteins of interest from crude preparations of biolo-



Continuous-elution electrophoresis cell accepts 100 ng to 50 mg of total protein.

gical tissues and fluids to single-band purity within 24 hours. In addition, Bio-Rad's Econo System functions as a useful accompaniment to the Prep Cell. This set-up provides pumping, collecting, monitoring and recording capabilities and, at the same time, adds extra purification capacity in the form of a complete chromatography workstation. See booths 124/1323.

With the introduction of a proprietary amino acid activation system, the new Model PS3 automated peptide synthesizer, developed by Protein Technologies and available from Rainin Instruments, is designed to provide high yields at low cost (*Reader Service No. 104*). The \$29,500 (US) Model PS3 is based on dry-activation Fmoc (9-fluorenylmethoxycarbonyl) chemistry, which significantly simplifies the instrument engineering that is required to create and deliver the highly reactive hydroxybenzotriazole (HOBt) ester to the reaction vessel, Rainin says. The Fmoc amino acids are prepacked with the activating agent benzotriazolyloxytris (dimethylamino) phosphonium hexafluorophosphate (BOP) in dry form. The dry mixture, which is stable indefinitely at room temperature, is activated by the addition of solvent. Pre-activating the amino acid



PS3: the three in one peptide synthesizer.

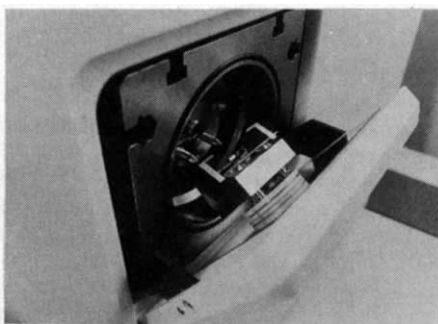
in the vial reduces by more than half the valves and plumbing required in the manufacture of the peptide synthesizer, which should result in a synthesizer that is more economical and more reliable, Rainin says. With the emphasis on productivity, the PS3 has three reaction vessels for the unattended synthesis of three different peptides. Typically, cycle times are only 32 minutes per amino acid. The Model PS3 provides wide programming flexibility for unusual syntheses; standard programmes can be used as templates to modify parameters such as coupling times, solvent volumes, deprotection times and washes. Customized programmes can be stored for later use. Rainin will be in booth 1034.

Screen from one to 24 antibodies against a single antigen on a blotted sheet with the new Milliblot-MP immunoassay processor from Millipore (*Reader Service No. 105*). The device is intended for dedicated assay configurations such as neonatal screening confirmation, hybridoma screening or other western blot assays. As the Milliblot-MP device accepts a single membrane sheet, there is no need to cut and handle individual membrane strips, says Millipore. Precise chambering and a spring-loaded interlock provide a compression fit. This set-up results in distinctly enclosed channels on the membrane and elevated reaction surfaces. This eliminates cross-talk between channels, the company says. The system is completely closed and does not require membrane transfer to complete the assay protocol. Conjugate and chromagen incubations can be carried out within the system. The Milliblot-MP, when used in conjunction with the Milliblot-SDE protein transfer system, provides users with a complete systems approach to blot transfer and processing. Number 1339 is Millipore's booth.

Limits of detection

The new LASERMAT mass analyser from Finnigan Mat will be put through its paces at the ACS meeting — see booth 300 (*Reader Service No. 106*). The instrument is designed for the accurate molecular weight determination of biopolymers

such as proteins, peptides and oligosaccharides from picomole amounts. LASERMAT has a wide mass range from 500 to in excess of 200,000 daltons. The combination of a straightforward sample loading procedure and Microsoft Windows 3.0 graphical user interface ensures that all laboratory staff, experienced and inexperienced staff alike, can operate the instrument. A solution of the sample to be analysed is mixed with a thousand-fold excess of matrix material and allowed to dry, forming a crystalline deposit on the LASERMAT sample slide. The slide is placed in the instrument's sample hatch and the system is evacuated. The sample-matrix deposit is then irradiated by a short pulse of laser light, causing the matrix to volatilize and produce intact molecular ions from the sample. These ions are accelerated by a strong electrical field down a flight tube. As all the ions have the same energy, lighter ions travel faster along the tube than heavier ions. The arrival of the ions at the detector is



LASERMAT mass analyser — see booth 300.

measured and the LASERMAT's software converts flight times to mass in a matter of seconds, displaying the results graphically, Finnigan Mat says. Production of a MASSMAP, or molecular weight map, enables LASERMAT to provide high sensitivity analysis of proteolytic digests, allowing screening with far greater specificity than HPLC-based mapping protocols, the company says. The instrument also provides routine confirmation of post-translational modifications to peptides and proteins, and verification of the structural integrity of synthetic peptides.

The latest addition to Varian's line of HPLC detectors is the new 9070 programmable scanning fluorescence detector (*Reader Service No. 107*). The 9070 is both a detector and a spectrofluorometer. The combination of scanning and wavelength programmability is designed to simplify optimization of the detector and provide maximum sensitivity. The instrument has been geared for maximum performance, with up to 24 time-wavelength programming steps per programme, Varian says. The menu-driven interface provides quick and easy programming of up to nine methods,

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which can be stored in the instrument's non-volatile memory. Excitation and/or emission scanning can be performed automatically, live-run or manually. Another design feature of the \$9,790 (US) 9070 detector is the pulsed Xenon lamp with dual settings — 100 Hz for measurement at trace levels and 20 Hz, which significantly extends the lamp life during routine analysis, Varian says. See booth 1107.

Molecular modelling

Molecular Simulations has launched Version 3.0 of POLYGRAPH, **polymer modelling software** developed specifically for the simulation, analysis and design of amorphous or amorphous/crystalline polymers and polymer blends (*Reader Service No. 108*). Version 3.0 includes faster algorithms that reduce substantially the amount of time it takes to simulate complex molecules, and a new X-windows-type user interface. In addition, Version 3.0 features five optional modules to help researchers analyse polymer structure using diffraction data, quantum mechanical calculations and

statistical mechanics. The five modules, CERIUS Diffraction Data, CERIUS Diffraction I and II, CERIUS StatMech and CERIUS MOPAC UI, were developed by Cambridge Molecular Design, Molecular Simulations' subsidiary, in association with Cambridge University. The first three of these form a package that allows scientists to utilize fully all the available data in the modelling or structural analysis process. CERIUS Diffraction Data performs orientation analyses on raw data from an X-ray diffraction experiment. CERIUS Diffraction I allows scientists to simulate the diffraction data that would result from a diffraction experiment on a proposed crystalline polymer, and CERIUS Diffraction II simulates diffraction patterns of amorphous polymers. POLYGRAPH Version 3.0 operates on 3-D workstations and supercomputers, including Silicon Graphics' IRIS, IBM's RS6000 and Stardent Computer's Titan 3-D workstations, and CRAY, Convex and Alliant supercomputers. Prices range from \$60,000 to \$90,000 (US), depending on the platform and graphics options. See booth 725.

At the ACS meeting, Polygen will be demonstrating a selection of new or upgraded software modules, including an **NMR software module**, a small molecule software module, an enhanced protein modelling module, and a protein structural refinement package — all of which

with experimentally-derived constraints, statistical analysis and display of multiple structures, and further refinement of structures through improved spectral assignment and relaxation matrix methods. See booth 1133.

For a limited period, Tripos Associates has slashed the price of its **computer-aided molecular design package** for bench scientists, called LabVision (*Reader Service No. 110*). Until 30 September 1991, the package, which is usually licensed for more than \$10,000 (US), will be offered at \$2,500 (US). Launched in late March of this year, LabVision is intended to meet the scientist's need for easy molecular visualization, discovery and presentation. Pull-down menus and dialogue boxes allow users to begin building models in minutes, Tripos says. LabVision allows the user to design proteins, carbohydrates and nucleic acids, and then view them in a variety of formats, including backbone ribbon, spacefill, and ball and stick. The package can be used to perform essential molecular computations, including molecular dynamics with animated playback, conformational searching, interface to MOPAC and interactive docking. For scientists wishing to try out the package before buying, Tripos offers a 30-day free evaluation period. LabVision operates on either stand-alone workstations (ESV, SGI 4-D Series or IBM RISC Station 6000), VAX/VMS, or on a network with Macintosh or PC graphics extensions. See Tripos in booth 1021. □

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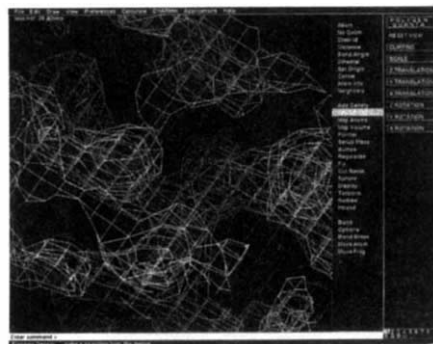
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Electron density maps from Polygen's protein structural refinement package.

run on the IBM RISC System 6000 and Silicon Graphics' platforms (*Reader Service No. 109*). In particular, QUANTA/NMR provides an integrated workbench of tools for spectral processing and analysis and molecular modelling for the spectroscopist. Connected by a common graphical interface, the tools in this workbench provide the spectroscopist with a molecular modelling system that is tailored to the process of structure determination. The module is designed to offer a complete solution for NMR spectroscopy, and features interfaces for off-line processing of NMR data, analysis of spectra for assignment, extraction of constraint information from NMR spectra, the generation of structures consistent

CORRECTIONS

Solid-phase gene assembly

Kenneth L. Beattie and Richard F. Fowler

Nature **352**, 548-549 (1991)

In the above product review in the 8 August issue, the sentence "A prototype instrument, operating at 0.1-1 μ mol scale, can produce 50-100 oligonucleotides within 3-6 hours, at a 30-50 per cent reduction in reagent scale consumption as compared to standard DNA synthesis instruments" was incorrect. It should have read "A prototype instrument, operating at 0.1-1 μ mol scale, can produce 50-100 oligonucleotides within 3-6 hours, at a 50 per cent reduction in reagent consumption as compared to standard DNA synthesis instruments" (third of way down third column, page 548).

Also, "(2) creation of libraries of duplex DNA segments encoding protein molecules..." should have read "(2) creation of libraries of duplex DNA segments encoding protein modules..." (two-thirds of way down second column, page 549).

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